APPROACH ON RESISTANCE STRATEGIES IN *STAPHYLOCOCCUS AUREUS*:

I CELL-MEMBRANE ASSOCIATED STEPS OF PEPTIDOGLYCAN SYNTHESIS

II TEMPORAL PATTERNS OF GLOBAL REGULATORS IN A *HEMB* MUTANT

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Dedicated to my parents

ZUSAMMENFASSUNG

Das Gram-positive Pathogen *Staphylococcus aureus* ist zwingend auf die korrekte Synthese des Peptidoglykanvorläufers Lipid II-Pentaglycin (LII-Gly_5) angewiesen. Eine komplette Blockierung der Produktion der Pentaglycinseitenkette ist letal, eine partielle Hemmung führt auch in Methicillin-resistenten *S. aureus* (MRSA) zu einer generellen Antibiotika-Überempfindlichkeit. Genetische Analysen haben ergeben, dass die Gly_5 Synthese sequentiell durch die Faktoren FemABX, nicht-ribosomalen Peptidyl-Transferasen, katalysiert wird. Um die molekulare Peptidoglykan-Biosynthese aufzuklären, wurde ein *in vitro* System entwickelt, welches die Testung verschiedener Kombinationen von Fem-Faktoren mit potentiellen Substraten erlaubte. Es zeigte sich, dass FemX, welches das erste Glycin anhängt, nur LII, FemA nur LII-Gly, und FemB nur LII-Gly_3 erkennt. Die Fem-Faktoren waren individuell aktiv und benötigten keine weiteren Proteine. Dieser Assay wird weiterentwickelt werden um potentielle FemABX-Inhibitoren zu identifizieren. Multiresistente MRSA, sowie weitere Humanpathogene wie Pneumokokken und Enterokokken, die ein verzweigtes Peptidoglykan haben, könnten mit Substanzen dieser neuen Antibiotika-Klasse kontrolliert und therapiert werden.

Abwehrmechanismen des Wirts und die Wirkung von Antibiotika können in *S. aureus* durch die Bildung so genannter small colony variants (SCVs) umgangen werden, deren Physiologie und Phänotyp atypisch sind. Durch Inaktivierung von *hemB* wurde eine stabile SCV konstruiert. Der Vergleich von Wildtyp und *hemB* Mutante zeigte signifikante Unterschiede in Bezug auf die Expression von Regulatoren und Virulenzfaktoren. In der *hemB* Mutante war die Transkription nach der exponentiellen Wachstumsphase allgemein drastisch reduziert. Überraschenderweise wurden aber SigB kontrollierte Gene, insbesondere *sarC* und *clfA*, während des gesamten Wachstums transkribiert. In einer *hemB sigB* Doppelmutante fehlten diese Transkripte, was auf deren Abhängigkeit von SigB und eine durchgehende SigB Aktivität in der *hemB* Mutante hindeutet. SigB ist somit einer der Regulatoren, die in der *hemB* Mutante die Expression von Virulenzfaktoren kontrollieren. Weil diese Studie in isogenen Stämmen und unter konstanten experimentellen Bedingungen durchgeführt wurde, stellen die generierten Daten eine bisher einzigartige Übersicht über die Transkriptionsprofile der verschiedenen Regulatoren dar.

SUMMARY

Accurate synthesis of the peptidoglycan precursor lipid II-pentaglycine (LII-Gly_5) is crucial for the Gram-positive pathogen *Staphylococcus aureus*. The absence of the pentaglycine chain is lethal; a partial inhibition of its synthesis conveys general antibiotic hyper-susceptibility, even to methicillin-resistant *S. aureus* (MRSA). Genetic analysis had revealed the sequential elongation of the Gly_5 chain to be catalysed by the non-ribosomal peptidyl-transferases FemABX. To elucidate the molecular basis of peptidoglycan biosynthesis, an *in vitro* system was developed for testing different combinations of Fem factors with their potential substrates. FemX, responsible for the addition of the first glycine, was found to recognize only LII, FemA only LII-Gly, and FemB only LII-Gly_3. None of them required the presence of any of the other Fem factors, or additional proteins. This assay will be further developed to possibly identify potential FemABX inhibitors. Multi-resistant MRSA and other human pathogens with a branched peptidoglycan, like pneumococci or enterococci, could be controlled and eradicated with substances belonging to this new class of antibiotics.

S. aureus can escape host defence as well as antibiotic-treatment by formation of so-called small colony variants (SCVs) among other strategies. SCVs display an atypical physiology and phenotype. A stable SCV was created by inactivating *hemB*. Comparison of the transcription profile of global regulators and virulence factors revealed significant differences between the parental strain and its *hemB* mutant. A general transcription stop was observed after the exponential phase in the *hemB* mutant. However, the reported target genes of SigB, *sarC* and *clfA*, were transcribed throughout growth. In a *hemB sigB* double mutant these transcripts were absent, indicating (i) their dependence on SigB and (ii) confirming the permanent presence of active SigB in the *hemB* mutant. While a potential influence of additional regulators can not be excluded, it was shown that SigB is involved in the control of the *hemB* transcriptome. Moreover, since this study was performed in one defined genetic background it provided a unique opportunity to interrelate transcriptional patterns of different regulators over the entire growth cycle.

1 INTRODUCTION

1.1. The pathogen *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium with low G+C content and high genetic plasticity. Its circular chromosome, of approximately 2.8 Mb, and plasmids, ranging from 1 – 60 kb, can harbour several pathogenicity islands, prophages, transposons and insertion elements, each conferring additional properties to the organism, especially in respect to resistance and virulence. Up to 20 % of the *S. aureus* genome can be constituted of such otherwise dispensable and accessory genes (13, 167, 216). The remarkable ability of *S. aureus* to rapidly adapt under selective pressure is in part explainable by the transfer of mobile elements between staphylococcal strains, species, and other Gram-positive bacteria (344).

S. aureus can be encountered in the environment as well as on its hosts; man and livestock. Human ecological niches of *S. aureus* are the skin and mucous membranes, where this opportunistic pathogen usually grows asymptomatically. Several studies reported 30 to 60 % of healthy adults to be transiently colonised by *S. aureus*, with 10 to 20 % persistently colonised (58, 202, 281). Carriers not only contribute to the distribution of *S. aureus* (203), they are also at increased risk for subsequent infections. Especially for immunocompromised individuals, intravenous drug addicts, patients undergoing surgery or on dialysis and those with implanted medical devices, carriage has been identified as an important risk factor for infection (202, 357, 395). Apart from rather harmless superficial skin infections and food poisoning, *S. aureus* can cause a wide range of severe illnesses, such as endocarditis, sepsis, osteomyelitis and pneumonia. *S. aureus*-induced disease is often suppurate and associated with extensive tissue destruction and necrosis.

The ability of *S. aureus* to survive harsh conditions and adopt several resistances has made its cure difficult. Recent reports indicate that the rates for methicillin-resistant *S. aureus* (MRSA) related infections are increasing (87, 147) and endogenous intermediate resistance against glycopeptides, which are often the antibiotics of last resort, has begun to develop (165, 329). In addition, the acquisition of the *vanA* gene from *Enterococcus faecalis* by *S. aureus*, mediating high-level vancomycin resistance, has been reported (405).

In the past, *S. aureus* reacted to the introduction of new antibiotics by developing resistance within one year (179, 314) and remains one of the leading causes of hospital- and community acquired infections. The consecutive spread of the resistant strains from the hospital to the community was a predictable event. Less expected was the emergence of novel strains of MRSA in the community, that are genetically distinct from MRSA strains originating in the hospital, as observed in the past decade (59, 81, 167, 271). These community-acquired MRSA (CA-MRSA opposed to HA-MRSA, for hospital-acquired) are often resistant against fewer antibiotics than HA-MRSA, and fitter, due to the lower genetic burden (83, 98). When entering hospitals, CA-MRSA can therefore replace HA-MRSA, contributing to an increased turn-over of MRSAs (287, 337). Proper susceptibility profiling and moderate use of antibiotics is strongly recommended to reduce fatal outcomes and the development of new (multi-drug) resistant strains (8, 89, 300).

1.2. Regulation of virulence

The controlled, appropriate and coordinate expression of virulence factors during infection is a prerequisite for a successful establishment in the host (Fig. 1). Overlapping and interacting regulatory networks consisting of a multitude of global regulators allow *S. aureus* to react to changing environments and to adapt to the specific niches encountered. A vast array of virulence factors assures survival in a cooperative way; many are redundant and none is essential.

Due to the elicited regulatory response imposed by a specific infection model and difficulties in standardising cultivation conditions in the host organism, *in vivo* expression analyses reveal often only part of the regulatory network and are prone to greater deviations. When considering *ex vivo* expression analyses of clinical isolates, one has to take into account that these strains likely harbour mutations and might not represent standard *S. aureus*. However, contrary to *in vitro* data, careful study of such adapted organisms gives valuable information about host-pathogen interaction, minimal required elements for a defined infection and possible bypass pathways in the case of mutations.

For standardising growth conditions and in order to reliably reproduce fluctuating changes, *in vitro* cultivation is recommended. Thereby, the growth phase dependent expression of first surface-associated and then excreted proteins is observed, representing the change from a colonizing to an invasive behaviour (312).

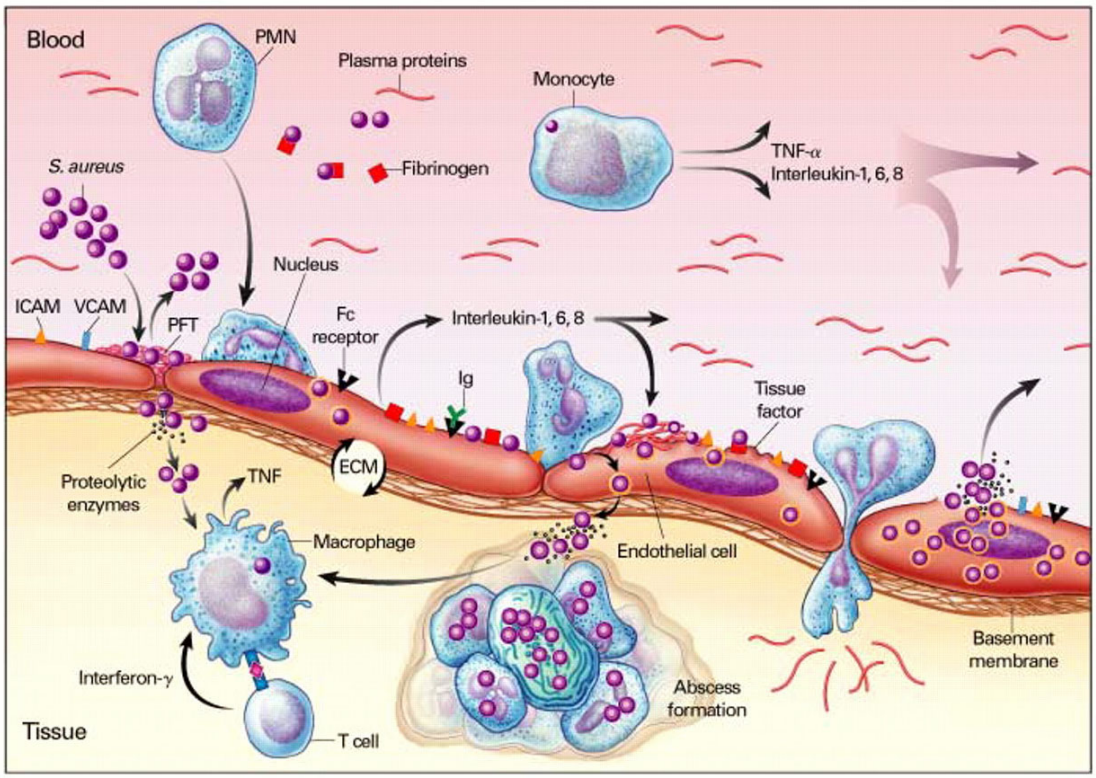
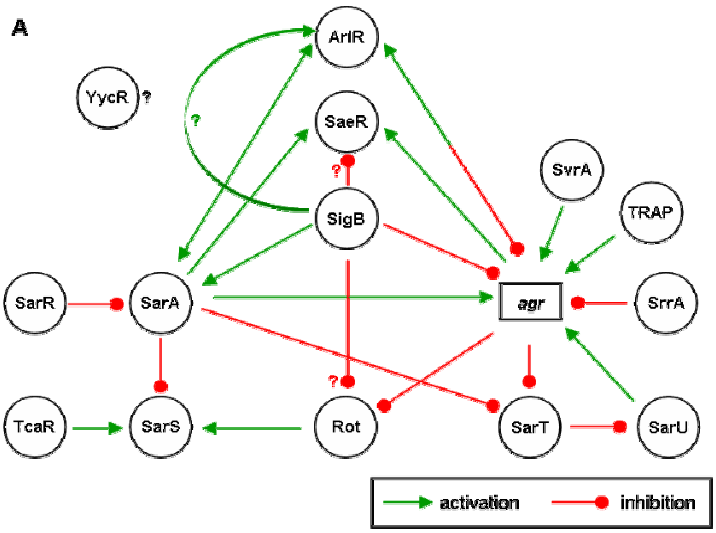


Fig. 1. Pathogenesis of staphylococcal invasion of tissue. The sequence of events progresses from left to right. Circulating staphylococci bind to sites of endovascular damage where platelet-fibrin thrombi (PFT) have formed. The bacteria may attach through MSCRAMM-mediated mechanisms. Alternatively, they may adhere to endothelial cells directly through adhesin-receptor interactions or by means of bridging ligands that include serum constituents such as fibrinogen. Modifications of the endothelium resulting from microenvironmental changes (such as alterations in the extracellular matrix (ECM)) can signal changes in cellular susceptibility to infection. After phagocytosis by endothelial cells, the bacteria elaborate proteolytic enzymes that facilitate the spread to adjoining tissues and the release of staphylococci into the bloodstream. Tissue factor is expressed by infected endothelial cells, facilitating the deposition of fibrin and the formation of vegetations. Once in the adjoining subepithelial tissues, the bacteria elicit an inflammatory response that results in abscess formation. This sequence of events contributes to the establishment of metastatic foci of infection, as well as the pathogenesis of endocarditis when cardiac endothelium is involved. After phagocytosis, endothelial cells express Fc receptors and adhesion molecules (vascular-cell adhesion molecules; VCAM, and intercellular adhesion molecules; ICAM) and release interleukin-1, interleukin-6, and interleukin-8. As a result, leukocytes adhere to endothelial cells, with diapedesis to the site of infection. Changes in the conformation of endothelial cells result in increased vascular permeability, with transudation of plasma proteins. Both tissue-based macrophages and circulating monocytes release interleukin-1, interleukin-6, interleukin-8, and tumor necrosis factor (TNF-α) after exposure to staphylococci. Macrophage activation occurs after the release of interferon-γ by T cells. Cytokines released into the bloodstream from monocytes or macrophages, as well as endothelial cells, contribute to the manifestations of the sepsis syndrome and vasculitis associated with systemic staphylococcal disease. Expression of Fc receptors may contribute to the vasculitis occasionally encountered during bacteremia by acting as a binding site for immunoglobulin (Ig) or immune complexes. PMN denotes polymorphonuclear leukocyte. Reproduced from (228).

A description of the interplay of regulators and their effect on selected virulence determinants follows and is schematically summarised in Fig. 2.



B

target	+ influenced by				- influenced by				
asp23		SigB			?				
clfA		SigB			?				
fnbA	SaeR	SarA	SigB		*agr*				
hla	SaeR	SarA	SvrA	*agr*	ArlR	SigB	Rot	SarS	
isaA	SarA	SigB	YycR ?		YycR ?				
spa	TcaR	Rot	SarS		SarA	ArlR	SrrA	SvrA	*agr*

Fig. 2. State of the knowledge of the interplay of global regulators and regulation of virulence determinants in *S. aureus.* **A** Regulatory network. Designated effects might be indirect and may occur at different levels of gene expression. **B** Target genes and factors influencing their expression.

1.2.1. Virulence determinants

Cell-associated products, including adhesins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family and capsular polysaccharide, facilitate binding to host tissue and help resist host immune responses. Most MSCRAMMs are covalently anchored to the cell wall peptidoglycan (described in section “Cell wall composition” and exemplified in Fig. 3 by a clumping factor). Secreted exoproteins, such as cytolysins or extracellular proteases, lipases and hyaluronidases, are thought to combat host defences and to facilitate tissue invasion, as well as to assure nutrient acquisition. Staphylococci have typically been regarded as non-invasive extracellular pathogens that damage host cells after adhering to the extracellular matrix; however, there is growing evidence that *S. aureus* has the ability to invade and persist within eukaryotic cells (4, 96,

178, 270, 316, 385). Following invasion of host cells, the release of cytokines is induced, exacerbating disease and triggering apoptosis (3, 46).

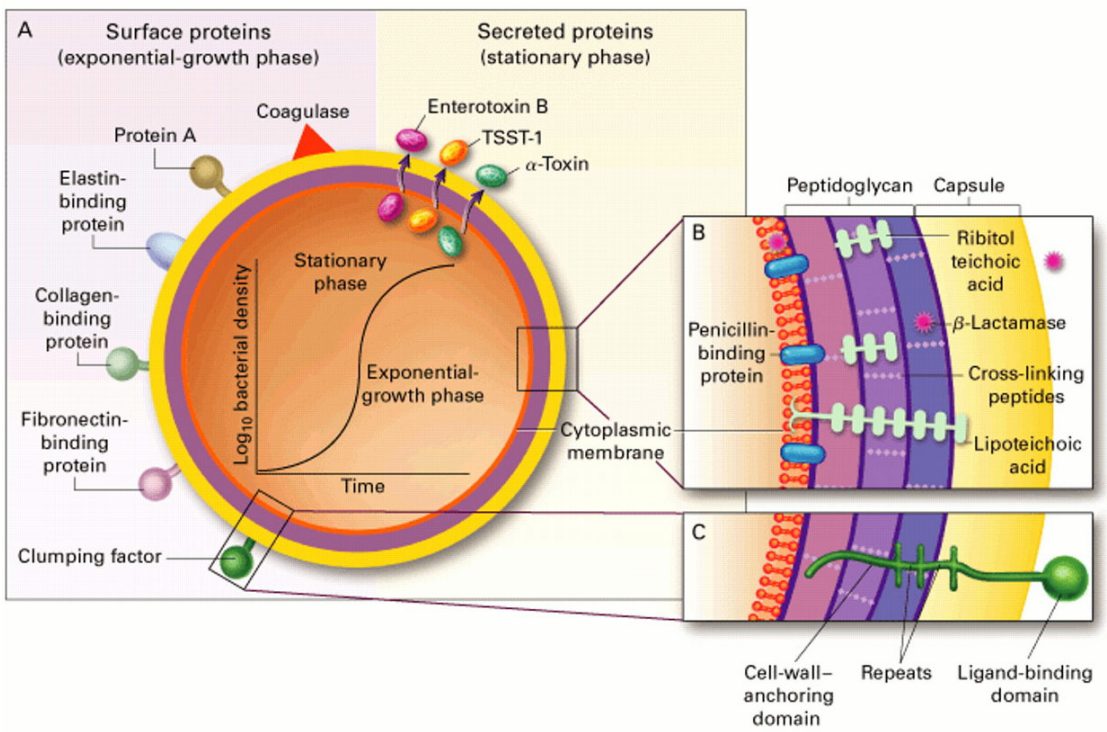


Fig. 3. Structure of *S. aureus*. Panel **A** shows the surface and secreted proteins. The synthesis of many of these proteins is dependent on the growth phase, as shown by the graph, and is controlled by regulatory genes such as *agr*. Panels **B** and **C** show cross sections of the cell envelope. Many of the surface proteins have a structural organization similar to that of clumping factor, including repeated segments of amino acids (Panel **C**). TSST-1 denotes toxic shock syndrome toxin 1. Reproduced from (228).

1.2.1.1. Adhesion factors (adapted from (3))

The ability to adhere to the extracellular host matrix and to interact with blood proteins is thought to be essential for colonisation and the establishment of infection (113, 227, 228). Moreover, the first step of host cell invasion is adhesion. Some host components targeted by *S. aureus* include fibrin(ogen), fibronectin, thrombospondin, von Willebrand factor, collagen, bone sialoprotein, and elastin (297). The first four proteins are found in the blood and adhere to indwelling catheters, based on studies in animals and human subjects. Implanted devices are generally quickly covered with bacteria if host cells and proteins are not fast enough in wrapping them up. Specific MSCRAMMs include fibronectin-binding proteins (FnBPs) (185), fibrinogen-binding proteins (44, 62, 254), elastin-binding adhesin (295), collagen-binding adhesin (296), and a broad specificity adhesin (MAP) that facilitates low-affinity binding of

S. aureus to several proteins, including osteopontin, collagen, bone sialoprotein, fibronectin, fibrinogen, and vitronectin (256). *S. aureus* can utilise MSCRAMMs to adhere directly to cells or via bridging ligands, cellular proteins with affinity for host receptors and MSCRAMMs (94, 351).

Fibronectin-binding proteins

FnBPs are likely the most-important MSCRAMMs in *S. aureus* colonisation, invasion of eukaryotic cells and establishment of human infections, including endocarditis (217, 356, 409). They are encoded by the two related and neighbouring genes *fnbA* and *fnbB*, which likely arose by gene duplication. Most clinical isolates contain at least one of them (264, 299). The interaction between *S. aureus* and host cells has been proposed to occur through a bridging model where fibronectin is bound by the FnBPs as well as by host cell integrins. Integrins mediate communication and adhesion between cells and are components of the extracellular matrix. A clustering of integrins, induced by ligand-binding, possibly causes activation of a signal transduction cascade leading to the expression of proinflammatory cytokines (46, 70, 73, 75). Interestingly, integrins have been proposed to be involved in receptor- mediated endocytosis of *S. aureus*. Apart from integrin, a mammalian heat shock protein has been shown to contribute to internalisation by interaction with FnBPs as well (94). Intracellular bacteria seem to trigger sooner or later apoptosis, whereby *agr* and *sar* regulators play a role. An exception of this rule are the small colony variants, described later.

Protein A

spa encodes the cell-wall associated protein A, which has a hypervariable repeat region binding immunoglobulin G (IgG) and C-reactive protein (76). Both host proteins are involved in opsonisation, phagocytosis and complement activation. Binding of the Fc domain of IgGs protects *S. aureus* against opsonophagocytosis (384). In addition, protein A binds to innate-like B-cells in the spleen, induces apoptosis and provokes B-cell depletion (144). Protein A displays high size heterogeneity among strains (53), but the number of repeats can also vary in one strain during prolonged infection (191). This is thought to be mainly caused by slipped-strand mispairing (SSM), which seems to occur in combination with inadequate DNA mismatch repair systems and is an important prerequisite for bacterial phase variation and adaptation (386).

Clumping factors

It is speculated that bacteria could protect themselves from phagocytic and immune defences by causing localized clotting. So far, *S. aureus* is known to encode three clumping agents, which in part also function as adhesins.

clfAB

Contrary to the FnbPs, the two fibrinogen-binding adhesins and clumping factors ClfA and ClfB are not encoded by allelic variants but distinct, unlinked genes. ClfB seems to be expressed only under aerobic conditions early in growth (278), while ClfA is predominantly present after the exponential phase (409). The interaction site of their products with fibrinogen differs too; ClfA binds to the γ chain of fibrinogen, while ClfB appears to bind rather to the α and β chains of fibrinogen. However, both proteins contain a Ser-Asp dipeptide repeat domain, serving as a stalk crossing the cell wall and presenting the A domain (Fig. 3), which binds the ligand in a Ca^{2+}-dependent way. The fact that the two Clf proteins recognise different parts of the host ligand, and might act synergistically to allow bacteria to attach more firmly to thrombi under flow conditions in the bloodstream, could explain why *S. aureus* possesses two fibrinogen-binding adhesins. Since ClfB also binds to keratin, thereby mediating adhesion to epithelial cells, it has been proposed to be required for nasal colonisation (402). ClfA has been shown to play an important role in the establishment of endocarditis. Recently, protection against phagocytosis by macrophages has been reported to be conferred by ClfA (293).

Coagulase

The secreted protein coagulase binds to and activates prothrombin, thereby causing clotting of platelets in a different way compared to the Clfs (410). The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory (9).

Toxins

Cytotoxins or -lysins, such as alpha-toxin (encoded by *hla*), form pores and induce proinflammatory changes in mammalian cells. The consequent cellular damage may contribute to manifestations of the sepsis syndrome (32, 401).

Superantigens are pyrogenic toxins which function by binding to major

histocompatibility complex class II proteins, thereby activating 10-50 % of the T-cell population (as compared to 0.01 % by processed antigens) and causing extensive T-cell proliferation as well as cytokine release (242). This overwhelming immune response leads to the toxic shock syndrome (TSS) characterised by fever, chills and vomiting, progressing to low blood pressure and multiorgan dysfunction (7, 97). Known proteins causing TSS are the enterotoxins and the structurally similar toxic shock syndrome toxin 1 (TSST-1), which is associated with most menstrual TSS cases and found in 20 % of *S. aureus* isolates. Enterotoxins are mainly responsible for food poisoning (154).

Exfoliative toxins, such as epidermolytic toxins A and B, cause skin erythema and separation, as seen in the staphylococcal scalded skin syndrome (SSSS). Both toxins produce blistering by disruption of the epidermal granular cell layer, apparently from direct effects on desmosomes. This disruption leads to interdesmosomal splitting. The toxins most likely bind directly to the desmosomal protein desmoglein-1, but the mechanism of action of the toxin is not fully understood.

Panton-Valentine leukocidin, a pore-forming toxin, has been epidemiologically associated with severe cutaneous infections, as well as pneumonia, and acts as a leukocytolytic toxin (74, 89, 194).

1.2.2. Global regulators

1.2.2.1. Two component regulatory systems

Two component regulatory systems are found in all prokaryotes, except mycoplasmas (118, 163), playing a critical role in sensing and responding to environmental conditions and in bacterial pathogenesis (166, 365). Generally, they consist of a sensor histidine kinase protein, which is usually bound to the bacterial membrane, and a cognate response regulator protein, which often acts as a DNA-binding transcription regulator. Autophosphorylation of the histidine kinase component in response to an environmental signal is followed by phosphoryl transfer to the response regulator, which alters the expression of regulon genes needed to respond to the environmental condition. 16 two component systems have been identified so far in *S. aureus;* only a subset is described here.

agr

The accessory gene regulator locus *agr* represses in post-exponential phase cell-surface associated proteins, such as *spa* or *fnbA*, and activates exotoxin production, e.g. *hla*

(175, 266, 286, 342). *agr* encodes two divergently transcribed mRNAs. RNAII transcription is initiated at the promoter P2, covering *agrBDCA* and coding for a quorum-sensing two-component system consisting of four elements (Fig. 4).

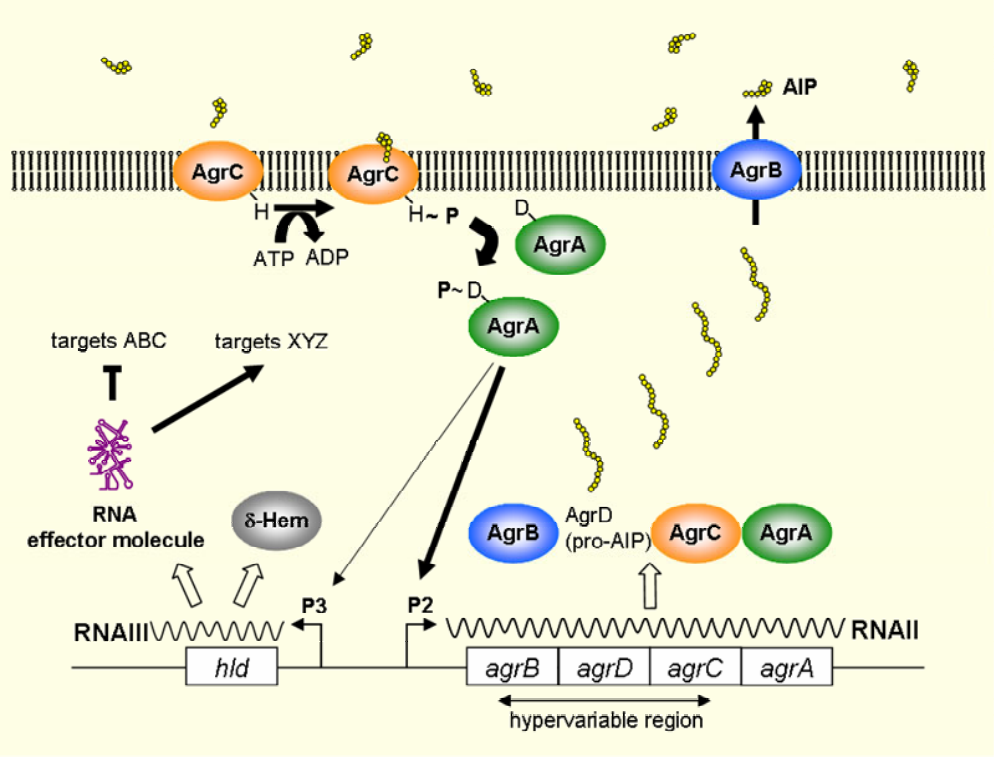


Fig. 4. Model of the *agr* system. The pro-AIP peptide is constantly processed and secreted by AgrB. The AIP binds to the receptor histidine kinase AgrC, activating auto-phosphorylation. In consequence, the response regulator AgrA is phosphorylated, up-regulating predominantly transcription from the P2 promoter of the *agr* locus (as symbolised by the thickness of the arrows). When reaching a certain threshold concentration of AIPs, RNAIII transcription is efficiently triggered as well. Besides encoding δ-hemolysin, RNAIII itself controls directly or indirectly, positively (arrow) or negatively (cross-bar) transcription of target genes. Below, a schematic organisation of the *agr* operon is given. Transcripts (wave lines), promoters (arrows) and open reading frames (boxes) are indicated. Proteins are represented by ellipses, peptides by bead-strings. The secondary structure of the regulatory RNAIII molecule (violet) is emblematic (adapted from (23)).

The propeptide AgrD is constantly N- and C-terminally processed and secreted to the medium with the aid of AgrB (180, 334, 422). Modified AgrD operates as an auto-inducing peptide (AIP) by binding to the receptor histidine kinase AgrC, thereby enhancing auto-phosphorylation and activation of AgrC (223). AgrC phosphorylates the LytTR family response regulator AgrA (280), which binds to paired heptad repeats in the P2 and P3 promoter regions of the *agr* locus. Phosphorylated AgrA has an increased affinity for P2, but not for P3, whose downstream direct repeat differs by 1 bp from the LytTR family consensus sequence (204). It is not yet clear whether AgrA binds the *agr* promoters as a mono- or as a

dimer. Known binding sites of SarA (280), positively regulating the expression of the *agr* locus (64, 68), partially overlap with AgrA protected areas (Fig. 5) (267, 315). Apparently, either physical displacement or interaction is necessary for *agr* regulation by both of these factors.

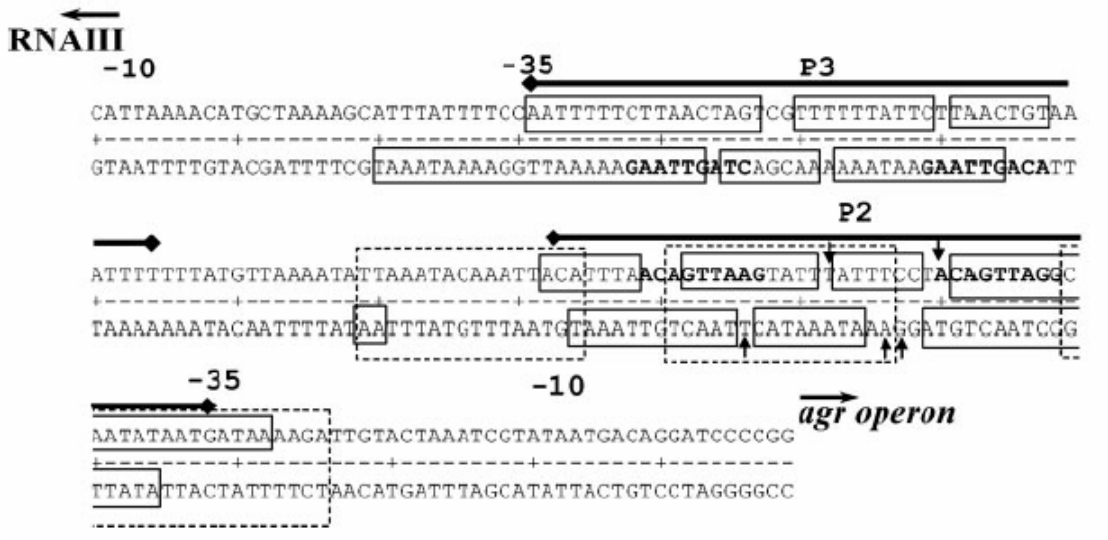


Fig. 5. Sequence of the RNAIII-*agr* intergenic region. The locations of the P2 and P3 promoter regions are indicated by the solid bars above the sequence. SarA-protected areas determined by Rechtin et al.1999 are shown by the dotted-line boxes (315). The direct repeats conforming to the consensus LytTR recognition sites are indicated in bold type. Protected areas of the P2-P3 promoter region are represented by solid boxes. The approximate locations of residues showing increased sensitivity to DNase I cleavage upon AgrA binding are indicated by vertical arrows. Adapted from (204).

Increasing cell density, and consequently AIP concentration, causes first RNAII amplification, leading to growing quantities of (phosphorylated) AgrA. After reaching high enough amounts, AgrA is able to trigger RNAIII transcription despite its lower affinity for P3. The threshold cell density for initiation of RNAIII transcription has been estimated to be 10^9 cfu/ml (15). Since a pattern search of the *S. aureus* genome using the AgrA consensus binding site revealed no other promoter sites, RNAIII has been proposed to exert the main effect of the *agr* locus as a regulatory molecule (286, 413). Its complex secondary structure (23) is well conserved among several staphylococci, although the sequence is not (24, 373). RNAIII has been shown to influence gene expression at the transcriptional and the translational level. While it has not yet been determined whether and how regulation of transcription occurs directly (23), translation of *hla* is favoured by pairing of the 5' RNAIII region with the *hla* leader sequence, which otherwise folds into an untranslatable configuration (266, 286). Another mechanism seems to be employed in the case of *spa*, where the translation initiation site has been proposed to be blocked by hybridisation to the

complementary 3' end of RNAIII (283).

The *agr* locus is conserved among staphylococci (90, 176, 180, 290). Based upon co-evolutionary variations in the hypervariable *BDC* region (Fig. 4), four *agr* specificity groups exist in *S. aureus*, which are defined by mutually inhibitory cross-group AIP-AgrC interactions. The resulting interference rather blocks the *agr* regulon than bacterial growth. Activation of the cognate receptor by the AIPs is highly sequence specific, e.g. type I and IV differ in but one amino acid (for a review see ref. (283)). Post-translational processing of AgrD leads to a 7-9 aa peptide, forming a characteristic, 5 aa thiolacton ring (also called macrocycle), with the N-terminal tail emerging at a conserved cystein (Fig. 6). This thiolacton ring is essential for AIPs, whether for inhibition or activation (230, 251, 255). Apparently, at the two C-terminal positions, bulky amino acids form a highly conserved hydrophobic patch, which has been proposed to be placed in a hydrophobic pocket of the receptor domain and to confer a general binding ability of AIPs to any receptor. The recognition of cognate AIPs by the polytopic receptor AgrC would then occur at specific contact sites of the proximal receptor end, influencing receptor responsiveness (413).

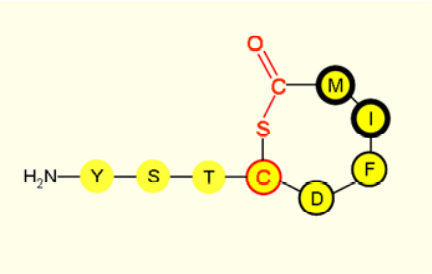


Fig. 6. Structure of the *agr* group I specificity autoinducing-peptide AIP. Amino acid residues are in yellow, those belonging to the macrocycle are encircled. The conserved, essential cysteine residue and the thiolactone bond are indicated in red. The C-terminal amino acids forming a bulky, hydrophobic patch are encircled by thick, black lines.

The interference between different *agr* groups might serve to isolate populations and may represent a major determinant of strain and species divergence. In *S. aureus*, *agr* groupings are broadly correlated with strain genotypes, resulting in general congruence between phylogenetic trees and *agr* groups. Thus, most menstrual toxic shock syndrome strains, and strains found to cause leucocidin-induced necrotizing pneumonia, belong to *agr* group III (132, 180). Most of the recently encountered glycopeptide intermediate resistant *S. aureus* (GISA) strains belong to *agr* group II (339, 392), and most exfoliatin-producing strains belong to *agr* group IV (176, 255). The cross-inhibitory activity of AIPs on a non-cognate *agr* regulon may bury a therapeutical potential; in a murine abscess model, administration of a synthetic group II AIP along with a group I strain strongly attenuated infection (251).

Although the importance of *agr* in virulence has been shown in disease models of skin abscess (251), endophthalmitis (45), septic arthritis (1, 42), endocarditis (66) and osteomyelitis (42, 131), where *agr* mutants displayed attenuated virulence, under certain

conditions *S. aureus* seems to use other regulatory circuits than *agr* to establish infection (137, 138). While an *agr* mutant can still cause pneumonia, invasive infection of the lung was no longer possible (161). In a peritoneal sepsis model, *agr* was not expressed and did not affect virulence (22). Similarly, in a device related subcutaneous infection model of guinea pigs and rabbits, *agr* expression was reduced compared to *in vitro* growth (138, 417). *Ex vivo* data from patients with cystic fibrosis revealed that *agr* expression was reduced in lung infection as well. Co-colonisation with strains belonging to different *agr* groups was consequently possible, which otherwise is rarely the case (139, 189). Possibly because it represents a metabolic burden (283), spontaneous *agr* mutants appear frequently in the laboratory (40, 358), biofilm or chronic infections (140, 416).

arl

The autolysis-related locus regulator/sensor protein (*arlRS*) plays a role in biofilm formation by influencing adhesion and autolysis. In addition, *arl* was found to be required for growth-phase dependent relaxation of DNA supercoiling, which positively influences *spa* transcription. Moreover, *arl* interacts with *sar* and with *agr* to modulate virulence factors like *spa* and *hla* (114-116). In strain Newman, but not in strain COL or in an $RsbU^+$ 8325 background, *arl* was found to be up-regulated by SigB (38).

sae

The *S. aureus* exoprotein expression locus *sae* has been shown to influence the production of several virulence factors with major consequences for infection, probably in response to various environmental stimuli (134, 136, 284, 363, 406). *sae* strongly depends on RNAIII in post-exponential phase (135, 284). While SarA has been reported to be a positive regulator, the effect of SigB on *sae* is not yet clear (363). In one case an attenuating effect had been observed in an 8325 background (284), whereas in a microarray study performed by our group in strain Newman, no such influence could be shown (38). *sae* is known to be required for *fnbA* and *hla* transcription (138). While *in vitro*, also *agr* and *sar* influence *hla*, *in vivo sae* is the predominant factor (138). For *fnbA* activation *in vitro*, *sae*, *sigB* and *sar* seem to be similarly important, since mutants of any of these regulators displayed reduced *fnbA* transcription (38, 272, 363, 411, 415).

srr

The auto-inducing *srr* locus (for staphylococcal respiratory response, *srrAB*; synonym staphylococcal *resDE* homologues, *srhSR*) reacts to environmental oxygen changes by

controlling expression of respiratory and fermentative enzymes as well as virulence factors, among which *agr*, TSST-1 and *spa* seem to be directly down-regulated (309, 375, 418). Under aerobic conditions, *srr* mutants grow normally, however, in the absence of oxygen, growth is impaired (418). Unpublished results of P. Schlievert showed that *srr* is down-regulated by *agr*, forming a cross-inhibition circuit (283).

yyc

yycFG (synonym *vicRK*) is the only known essential two-component system; its conditional down-regulation increases cell permeability as well as susceptibility against macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (245). Interestingly, the sensor histidine kinase YycG contains a PAS/PAC domain. PAS is an acronym formed from the names of the proteins in which the imperfect repeat sequences, accounting for the PAS domain, were first recognized: the *Drosophila* period clock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM). Situated carboxy-terminally to the PAS repeat, and contributing to the PAS structural domain, lies another similar region; the PAC motif (155). Unlike many other sensory domains, PAS domains are located in the cytoplasm where they function in various proteins as an input module monitoring changes in oxygen, redox potential and the intracellular energy status (371). Akin to most Gram-positive bacteria, *S. aureus* YycG has, in combination with the downstream encoded YycH and YycI components plus an aerobic terminal oxidase, the capacity for electron transport (277).

While its exact function remains to be resolved, the response regulator YycF has been shown to bind specifically the promoter regions, among others, of the antigens *isaA* and *ssaA*, but also of the *lytM* peptidoglycan hydrolase (86). Whether the proposed direct regulation is of positive or negative nature remains to be determined, however, overexpression of SsaA restores hypersensitivity to MLS_B antibiotics in a *yycF* mutant to wild-type levels (244). Therefore, *ssaA* regulation by YycF is most likely positive. A cell wall hydrolysing activity for the mainly secreted IsaA has been suggested, due to similarity with the soluble *E. coli* lytic transglycosylase and localisation of IsaA to the septal region of dividing cells (338). None of the so far identified targets in *S. aureus* are essential. A recent report about the *yyc* homologue in *Streptococcus pneumonia* identified this regulator to up-regulate fatty acid synthesis genes and to inhibit both the arginine deiminase *arcA* and the ornithine carbamoyltransferase *argF*, involved in arginine degradation and biosynthesis, respectively (Fig. 26) (263). In *B. subtilis*, *yycFG* might directly modulate the expression of the teichoic acid biosynthesis genes *tagAB*/*tagDEF* and positively influences transcription of the essential cell division operon *ftsAZ*, which is however also controlled by SigA and SigH

(120, 171). Multifactorial control of gene expression complicates the identification of crucial target genes not only of YycF. This regulator most likely influences further, yet unidentified, possibly essential genes in *B. subtilis* and in *S. aureus*, whose combined aberrant expression leads to cell death.

1.2.2.2. SarA-homologues

The vast SarA protein family can be divided in two subfamilies: i) single-domain factors (SarA, SarR, SarT, Rot) and ii) two-domain factors (SarS, SarU), with each domain sharing sequence similarity to the single domain factors (reviewed in (63)). Single-domain factors have been shown to dimerise, whereby they acquire a similar overall structure as two-domain factors (Fig. 7) (221, 225) and resemble the one of winged-helix family transcription factors as reported for eukaryotes (121).

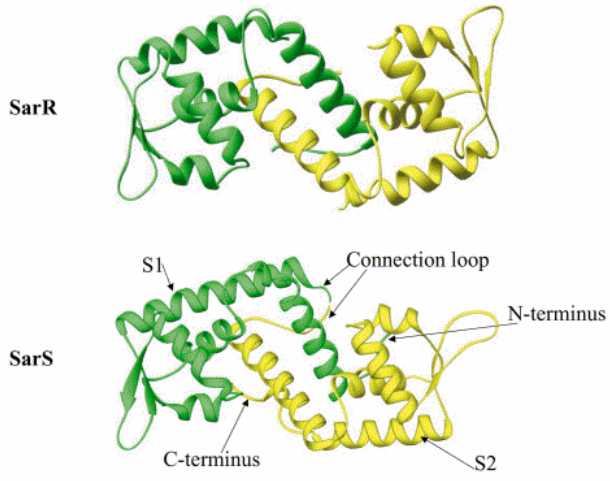


Fig. 7. Comparison of overall structures from SarR and SarS. The SarR structure is composed of two identical monomers, one in green and the other in yellow. In contrast, SarS is composed of two homologous but non-identical halves (S1 and S2) connected by a linker region. Both are winged-helix structures similar to the winged-helix proteins of eukaryotes. Reproduced from (63).

A central helical core is flanked by two winged-helix motifs. Within each winged-helix motif is a helix-turn-helix (HTH) motif and a β-hairpin turn, both putative DNA-binding domains (225). Conserved, basic amino acids are located on the concave surface, supposedly mediating DNA-binding (Fig. 8). Contrary, the convex side, functioning as the activating domain, displays a much higher diversity, reflecting the divergent functions of the SarA family members.

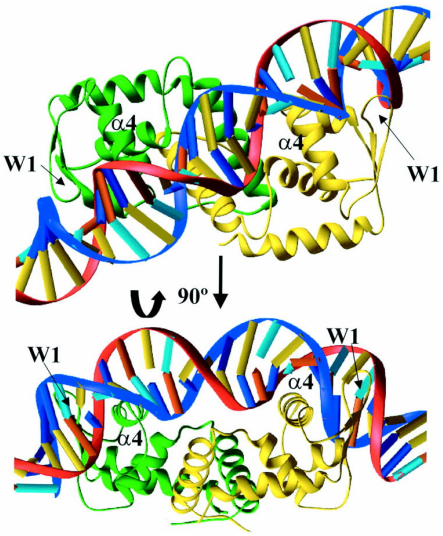


Fig. 8. The DNA binding model of SarR and DNA. The α4 helix and the wing region (W1) interact with DNA at major grooves and minor grooves, respectively. W1 conformation is slightly adjusted to fit in the minor groove. Reproduced from (225).

rot

Rot is a *sarS* activator, but has first been identified as an inhibitor of protease and α-toxin expression, for which it was named repressor of toxins (*rot*) (258, 336). Although transcription seems to be synergistically activated by *agr* and *sar*, *rot* is possibly post-transcriptionally repressed by *agr* (381). While in the latter report a negative influence of SigB on *rot* was found in an 8325 derivative, microarray analyses performed in our group could not confirm these findings in a Newman background (38).

sar

Three overlapping transcripts are expressed from the staphylococcal accessory regulator A locus (*sar*). *sarB*, *sarC* and *sarA* share identical 3' ends and all code for the global regulator SarA (Fig. 9), which regulates positively or negatively, directly or via other regulators, the expression of virulence determinants like *agr*, *fnbA*, *spa* and *hla*. Activators and temporal patterns of the individual *sar* transcripts differ. *sarB* and *sarA* are both most abundant in exponential phase and taper off towards post-exponential phase, their transcription is SigA-dependent. Instead, *sarC* transcription is selectively promoted by the alternative sigma factor SigB and increases towards stationary phase (21, 39, 65, 80, 122, 239). Indications for an involvement of the non-SarA-coding regions in post-transcriptional modulation of SarA activity exist. For instance, the *sarB* transcript contains elements located upstream of the SarA coding sequence which seem to be important for full SarA activity (64, 157). In the case of ORF3, a small additional coding region contained in *sarB* as well as in *sarC*, the nucleotide sequence itself has been reported to be essential for SarA-mediated *fnbA* activation, but not *spa* inhibition (411). Despite the diverse transcription, overall SarA protein levels have been reported to be rather constant during growth (41, 43, 170).

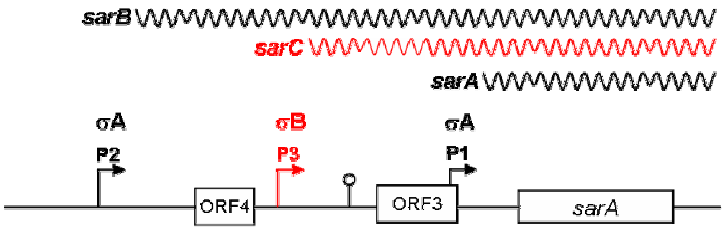


Fig. 9. Schematic organisation of the *sar* locus. Transcripts (wave lines), promoters (arrows), open reading frames (boxes) and an inverted repeat (hairpin loop) are indicated. The corresponding sigma (σ) factors initiating transcription are given above each promoter. Adapted from (239).

sarR

sar expression is modulated by SarR, which has been shown to bind to all three *sar* promoters. Interestingly, transcription from the SigB-dependent *sar* P3 promoter was reduced in a *sarR* mutant, while transcription from the strongest *sar* promoter P1 was enhanced. SarA levels were increased, as was consequently *agr* transcription (238, 239).

sarS

The staphylococcal accessory regulator S (*sarS*; synonym *sarH1*) binds to the promoter region of *spa*, thereby up-regulating transcription (122, 372). Contradictory results exist about SarS being a strong *hla* repressor (346, 372). *sarS* transcription is repressed by SarA and *agr*, but activated by Rot, SarT and TcaR (122, 253, 346). In an 8325-4 background, overproduction of SigB triggered *sarS* transcription initiated at a SigB-promoter consensus sequence; the resulting mRNA was paralleled by a possible processing product (372). However, in a Newman background, *sarS* transcription was not affected by SigB (38).

sarT

sar and *agr* repress *sarT* expression. SarT in turn down-regulates the *agr* activator SarU, thereby indirectly repressing *hla* transcription (240, 347). On the other hand, SarT up-regulates SarS, which positively influences *spa* expression (346).

sarU

Adjacent to *sarT*, but divergently transcribed, lies *sarU*. This *agr* activator might together with *sarT* represent an additional *agr* amplification loop, where increased *agr* activity decreases the expression of the SarU repressor SarT, thereby increasing *agr* activation by SarU (240).

1.2.2.3. Sigma factors

Initiation of transcription is the most important step of gene regulation. The single RNA polymerase core enzyme, engaged in all transcriptional activity, requires an additional subunit, designated as the sigma factor, to recognise promoter sequences and to initiate site-specific transcription (reviewed by (412)). Most bacteria synthesise several sigma factors, each of which recognises different consensus promoter sequences. This heterogeneity is the primal transcriptional regulatory mechanism, allowing the constitutive or conditional expression of physically unrelated genes that share the same promoter sequence. Unlike well-studied bacteria such as *Escherichia coli* and *Bacillus subtilis*, which have seven and 18 sigma factors, respectively, in *S. aureus*, besides the housekeeping sigma factor A, only two alternative sigma factors have been identified.

sigH

The over-expression of this recently identified sigma factor resulted in a drastic induction of the expression of the *com* operons that encode proteins required for the natural genetic competence (268). Since *S. aureus* does not form spores, the genetic competence is thought to be even more significant for the single cell survival and/or for the evolution of the species, because the imported DNA can serve as a nutrient, a template for DNA repair, and/or a supplier of genes that benefit cells (85). Under which conditions *S. aureus* is genetically competent is still unknown.

sigB

SigB, a regulator mediating stress response against UV, heat, acid and hydrogen peroxide strain (61, 126, 129, 170, 213, 214), also plays a role in antibiotic resistance as well as biofilm formation (37, 269, 313, 355, 414). Recently, a murine model of septic arthritis and a rat model of experimental endocarditis revealed SigB to be crucial for virulence (100, 183), whereas in other infection models SigB seemed not to be required (61, 170, 279). SigB had been shown to modulate directly or indirectly over 250 genes, among which *sar*, *hla*, *fnbA* and *clfA* (38, 65, 100, 127, 129, 272, 279, 318).

The *sigB* operon comprises apart from *sigB* itself, three regulators of sigma B (*rsb*) which control SigB activity in response to environmental stimuli on the post-translational level, and two smaller genes, SAS067 and SA1873, encoding hypothetical proteins. RsbU, RsbV and RsbW control SigB activity at the post-translational level (129, 262, 291). In the general model of SigB regulation (Fig. 10), derived from findings in *B. subtilis* (25, 158, 408),

the anti-SigB factor RsbW can form mutually exclusive complexes with either SigB or its antagonist, RsbV. Under normal conditions, RsbV is phosphorylated by RsbW. This modification impedes the formation of a stable RsbV-RsbW complex. The majority of RsbW can therefore aggregate to SigB, thereby hindering SigB interaction with the RNA polymerase core-enzyme (RNAP) and consequently transcription of genes preceded by a SigB consensus promoter sequence. Under stressed conditions, the RsbV~P-specific phosphatase activity of RsbU becomes activated by a yet unknown mechanism, dephosphorylates the anti-anti-SigB factor RsbV, which is thereby enabled to interact with RsbW. Consequently, the amount of RsbW-SigB complexes is decreased in favour of RsbW-RsbV aggregates, leading to free SigB, which then can form a holoenzyme with the RNA polymerase and direct transcription from target genes. The consensus promoter sequence for SigB targets was found to be GttTaa(N_{12-15})gGGTAt (127, 169).

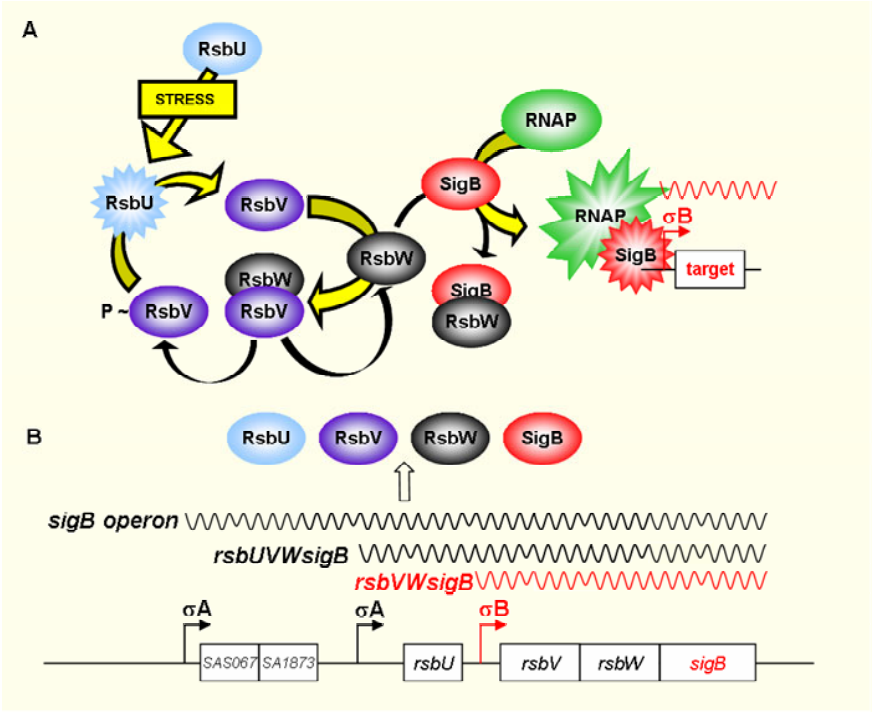


Fig. 10. Model of SigB activity regulation (**A**, adapted from (37)) and schematic organisation of the *sigB* operon (**B**). **A** The anti-SigB factor RsbW can form mutually exclusive complexes with either SigB or its antagonist, RsbV. RsbV is normally phosphorylated by RsbW (RsbV~P), which hinders it to complex RsbW, leaving the latter free to interact with SigB. When bound to RsbW, SigB can not aggregate with the RNA polymerase core enzyme (RNAP) to form an active holoenzyme. Upon stress, the RsbV~P-specific phosphatase activity of RsbU becomes activated and recycles RsbV~P to RsbV, favouring RsbW-RsbV aggregates and decreasing the amount of RsbW-SigB complexes. Freed SigB is then able to form a holoenzyme with RNAP and to direct transcription from its target genes. Activated proteins are star-shaped, yellow arrows designate reactions taking place under stressed conditions. **B** Transcripts (wave lines), promoters (arrows) and open reading frames (boxes) are indicated. The corresponding sigma (σ) factors initiating transcription are given above each promoter. Translation of *SAS067* and *SA1873,* encoding hypothetical proteins, is not represented.

While in *B. subtilis* an additional, upstream module exists, receiving environmental inputs leading to RsbU activity regulation, in *S. aureus* the corresponding proteins are missing (213, 414). A further RsbV phosphatase, RsbP, which has been proposed to sense energy stress, is absent as well (394). SAS067 and SA1873 share similarity with the *E. coli* "addiction module" systems (for a review see (99)) *mazE-mazF* and *pemI-pemK*, mediating programmed cell death. MazF and PemK are sequence-specific endoribonucleases that cleave mRNAs to inhibit protein synthesis, whereas MazE and PemI block their endoribonuclease activity (421, 423). The function of SAS067 and SA1873 in *S. aureus* remains to be determined.

1.2.2.4. Other regulators

TRAP/RAP

A second quorum sensing system has been proposed to exist, consisting of an RNAIII-activating protein (RAP) and the target of RNAIII-activating protein (TRAP). Similarly to the AIP of the *agr* quorum sensing system, the 277 aa RAP, which is identical with the ribosomal protein L2, functions as an autoinducer. RAP has been proposed to be somehow constantly secreted in *S. aureus*, although not containing a classical signal peptide (208). TRAP is phosphorylated at three conserved histidines in a RAP-dependent manner (14, 145) and has been found to be up-regulated in cells exposed to oxacillin treatment (354). By yet unknown mechanisms, phosphorylated TRAP activates *agr* and is itself indirectly dephosphorylated by AIP (14). TRAP seems to be membrane associated and at least in part to be extracellular. The lack of a kinase domain and a transmembrane region suggests the TRAP/RAP system to belong to a novel class of signal transducers and that additional factors might be involved. This model has been strongly debated due to apparent difficulties in reproduction of the isolation of RAP and its activating potential on *agr* (285). However, recombinant RAP has recently been shown to activate *agr* transcription (208). Although the significance of an extra-ribosomal function remains to be determined, it seems not be unique to *S. aureus*. For example, the ribosomal protein L11 from *Helicobacter pylori*, lacking any signal peptide, has been reported to be secreted as well (199).

svrA

The staphylococcal virulence regulator A (*svrA*) is a putative membrane associated protein of approximately 52 kDa, which is required for *agr* expression and virulence, as shown in a mouse model of peritoneal infection (124).

tca

The teicoplanin-associated locus regulator *tcaRAB* influences teicoplanin and methicillin resistance by a yet unknown mechanism. *tcaR* belongs to the MarR-like regulators and activates *sarS* transcription, while attenuating whole-length transcription of the cell-wall associated, putative adhesion protein *sasF* and inhibiting transcription of the intercellular adhesin *ica* locus, playing a key role in biofilm formation (177, 253). *tcaA*, a hypothetical transmembrane protein with potential sensor and/or signal transducer function, is thought to be involved in cell wall metabolism. Its inactivation leads to elevated glycopeptide resistance. *tcaB* encodes a membrane-associated protein with similarity to a bicyclomycin resistance protein present in several Gram-positive bacteria (50). Transcriptional regulation of the *tca* locus is complex and assignment of the individual bands is ongoing (236).

1.3. Resistance

Development of resistance against noxious substances encountered in the environment has always been part of bacteria's evolution. Nowadays, *S. aureus* with resistance against several antibiotic classes, disinfectants, antiseptics and heavy metal compounds exist (298). Concerns of a potential widespread of multidrug-resistant *S. aureus* is reinforced by the isolation of a multiresistance conjugative plasmid, combining a variety of resistance markers (405). Basic mechanisms conferring resistance are modification of the target, export, exclusion, inactivation and sequestration of the agent or bypass of the affected pathway; some representative, not exhaustive examples follow.

The *erm* genes, which are transposon-borne, encode rRNA methylases that modify, and thereby protect, the ribosomal site that is critical for the binding of group B streptogramins, macrolides and clindamycin (181).

Fluoroquinolones are readily separated from their site of action on DNA gyrase and topoisomerase IV by mutational up-regulation of NorA-mediated efflux (288).

Aminoglycosides can be inactivated by several cellular enzymes, such as acetyltransferases, adenyltransferases and phosphotransferases (353).

Trimethoprim, an analogue of the pteridine component of dihydrofolic acid, has a high affinity for dihydrofolate reductase. It thereby blocks the essential regeneration of dihydrofolate to tetrahydrofolate (THF) by the enzyme. THF can be converted to methylene-tetrahydrofolate, a methyl-donor for example in the conversion of dUMP into dTMP. Resistance is conveyed by an altered dihydrofolate reductase with reduced affinity for the drug (419), bypassing the blocked essential step in formation of thymidine, purines, methionine and glycine (275).

Both the antibacterial and DNA-cleaving activities of bleomycin are quenched by the product of *ble*, which has a high affinity for this antimicrobial agent (215).

Phenotypic variants with a reduced membrane potential display a decreased uptake of cationic antibiotics, such as aminoglycosides or host defensins, resulting in an increased resistance against these substances. Usually, different threshold levels are required for each substance (20). These so-called small colony variants are discussed later.

1.3.1. β-Lactam resistance and PBPs

All β-lactams contain an essential four-membered lactam (azetidinone) ring that can be fused to form bicyclic ring structures, as exemplified by penicillin G (Fig. 11). Their structural analogy to the D-Ala_2-end of the peptidoglycan precursor stem peptide (Fig. 12)

confers high affinity for the extracellular enzyme(s) usually catalysing the incorporation of the peptidoglycan precursor into the existing cell wall, for which proteins belonging to this class were named penicillin-binding proteins (PBPs) (377, 383).

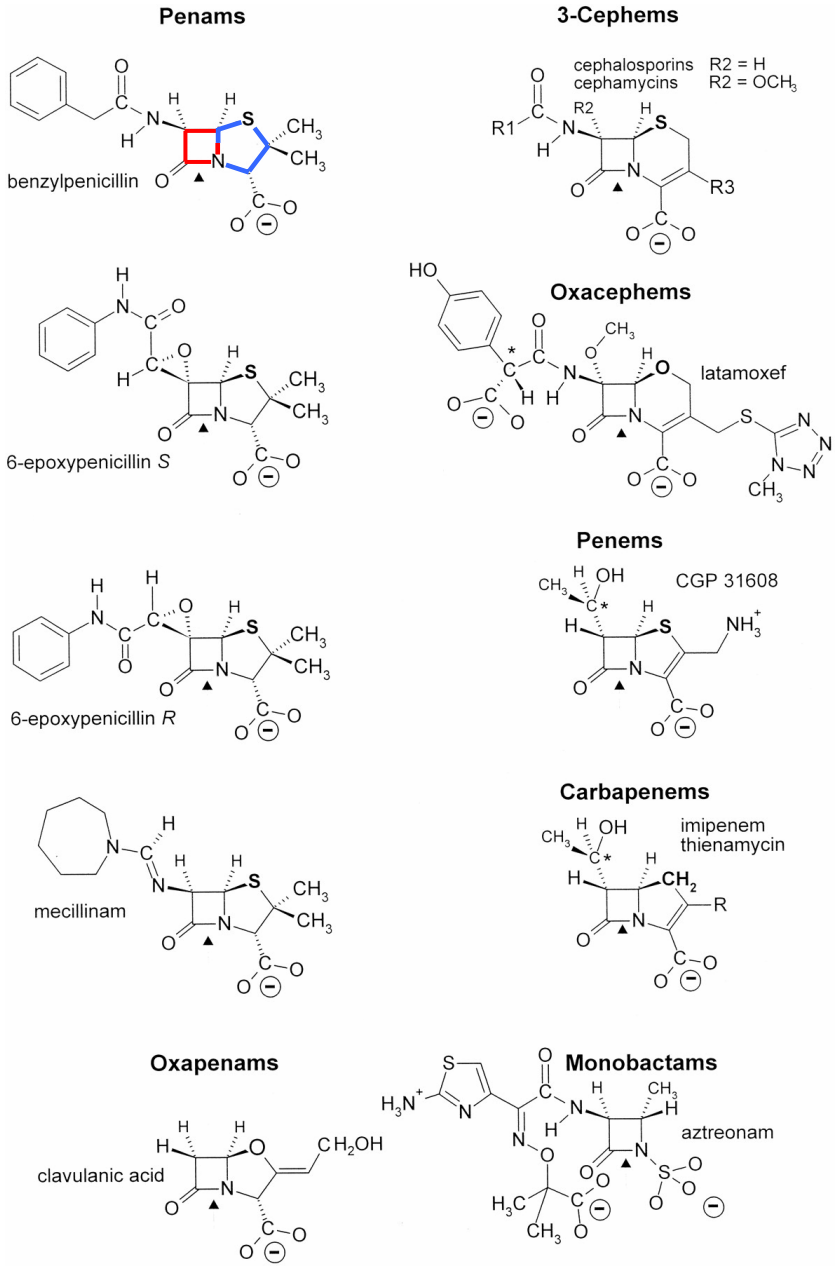


Fig. 11. Structures of β-lactams. In benzylpenicillin (synonym penicillin G), the characteristic bicyclic core, consisting of the β-lactam (red) and the thiazolidine ring (blue), is highlighted. Arrow heads indicate scissile bonds. Reproduced with minor changes from (141).

The first steps of Michaelis complex and acyl intermediate formation are similar to the transpeptidase reaction (A/B1→ A/B2 in Fig. 12). However, hydrolysis of the acyl-PBP intermediate is very slow (reaction B2→B3 in Fig. 12). Thus, PBPs are *de facto* irreversibly inactivated by β-lactams (141, 222). The lack of functional PBPs leads to reduced cross-

linking of peptidoglycan and consequently to a weakened cell wall, followed eventually by cell lysis and death (128).

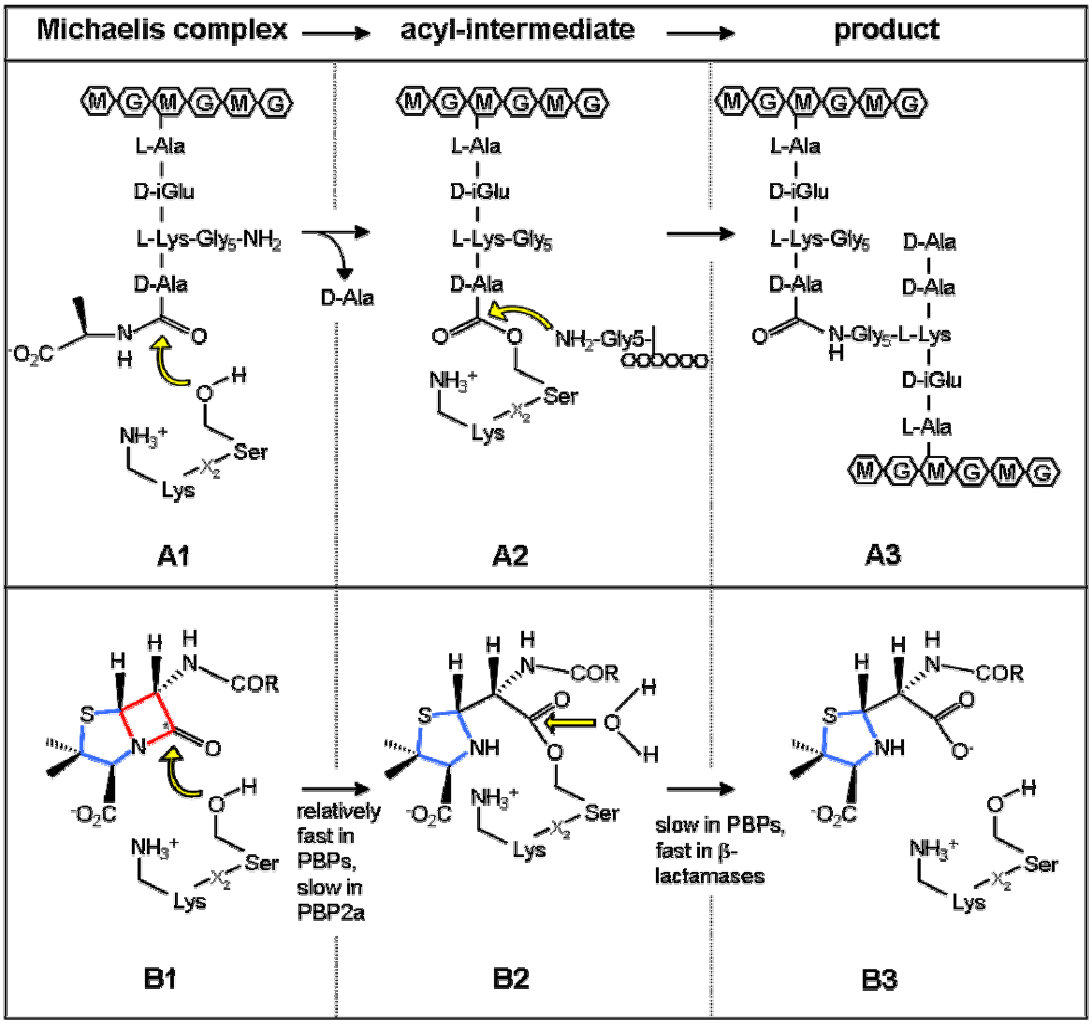


Fig. 12. A The transpeptidase reaction catalysed by PBP2. **A1** Nucleophilic attack of the active-site serine residue on the D-Ala$_2$ peptide bond. **A2** Formation of a covalent acyl intermediate, which can again be split by a nucleophile, here the amine of the pentaglycine linker. Alternatively, hydrolysis by water results in a net cleavage of the terminal D-Ala, leading to an uncrosslinkable tetrapeptide side chain. This reaction corresponds to the D,D-carboxypeptidase activity of PBPs, modulating the degree of cross-linkage. **A3** The new amide bond cross-linking two peptidoglycan chains. **B** Hydrolysis of penicillin catalysed by β-lactamase. **B1** Nucleophilic attack of the active-site serine residue on C* of the β-lactam ring. **B2** Penicilloyl intermediate, which is hydrolysed by water. **B3** Penicilloate, lacking anti-bacterial activity. Nucleophilic attacks are not given in detail but symbolised by yellow arrows. Only the S-X-X-K motif of the acetyltransferases is represented. As in Fig. 11, the β-lactam (red) and the thiazolidine ring (blue) are indicated. M, N-acetylmuramic acid; G, N-acetylglucosamin. Adapted from (55).

1.3.1.1. Penicillin resistance

The first resistance mechanism against classical β-lactams, such as penicillin, was inactivation by β-lactamase, a serine protease that is capable of fast hydrolysis of the β-lactam ring (reaction B2→B3 in Fig. 12). Usually, the structural gene for staphylococcal β-lactamase, *blaZ*, is located on a plasmid, together with regulatory elements controlling the expression (153, 282).

Despite an almost nonexistent similarity in their overall amino acid sequence, and different functions, PBPs form together with β-lactamases as well as β-lactam sensors the SxxK superfamily comprising Gram-negative and -positive bacterial acyltransferases. A common feature of these enzymes is a specific bar code in the form of three motifs, SxxK, (S/Y)x(N/C) and (K/H)(T/S)G, occurring at equivalent places and with roughly the same spacing along the polypeptide chains. In the three-dimensional structures, they are brought close together, forming the catalytic centre (Fig. 13) (141).

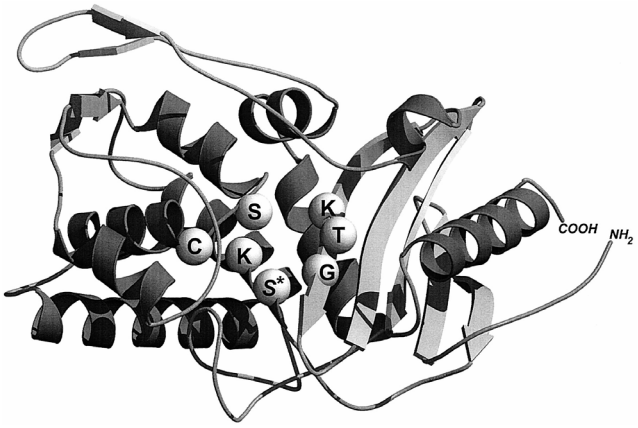


Fig. 13. Basic polypeptide fold of the acyltransferases of the SxxK superfamily and spatial disposition of the three catalytic center-defining amino acid groupings. The structure shown is that of the 262-amino-acid D, D-transpeptidase-PBP of *Streptomyces* sp. strain K15. The invariant motif 1, S*xxK, where S* is the essential serine nucleophile, motif 2 (S/Y)x(N/C); here SxC, and motif 3 (K/H)(T/S)G are depicted by spheres. Reproduced from (141).

The multi-modular group I acyltransferases are implicated in peptidoglycan synthesis. An N-terminal, hydrophobic segment, functions as both a signal sequence for secretion and a stop transfer signal that serves as a membrane anchor (Fig. 14). Afterwards, a non-penicillin binding (nPB) region follows, which in class A members corresponds to a glycosyltransferase module, and in class B members builds a linker region. The C-terminal acyltransferase, common to both classes, is then linked by an intermodule junction to the previous module, and, like those, has (sub-) class-specific motifs. Class A enzymes are responsible for the incorporation of the lipid II (LII) borne precursor peptidoglycan components into nascent peptidoglycans. Class B enzymes control wall expansion, ensure cell shape maintenance, direct and carry out septum formation as well as cell division (82, 241). This function is in part conferred by the nPB module mediating protein-protein interactions critical for peptidoglycan assembly in a cell cycle–dependent manner.

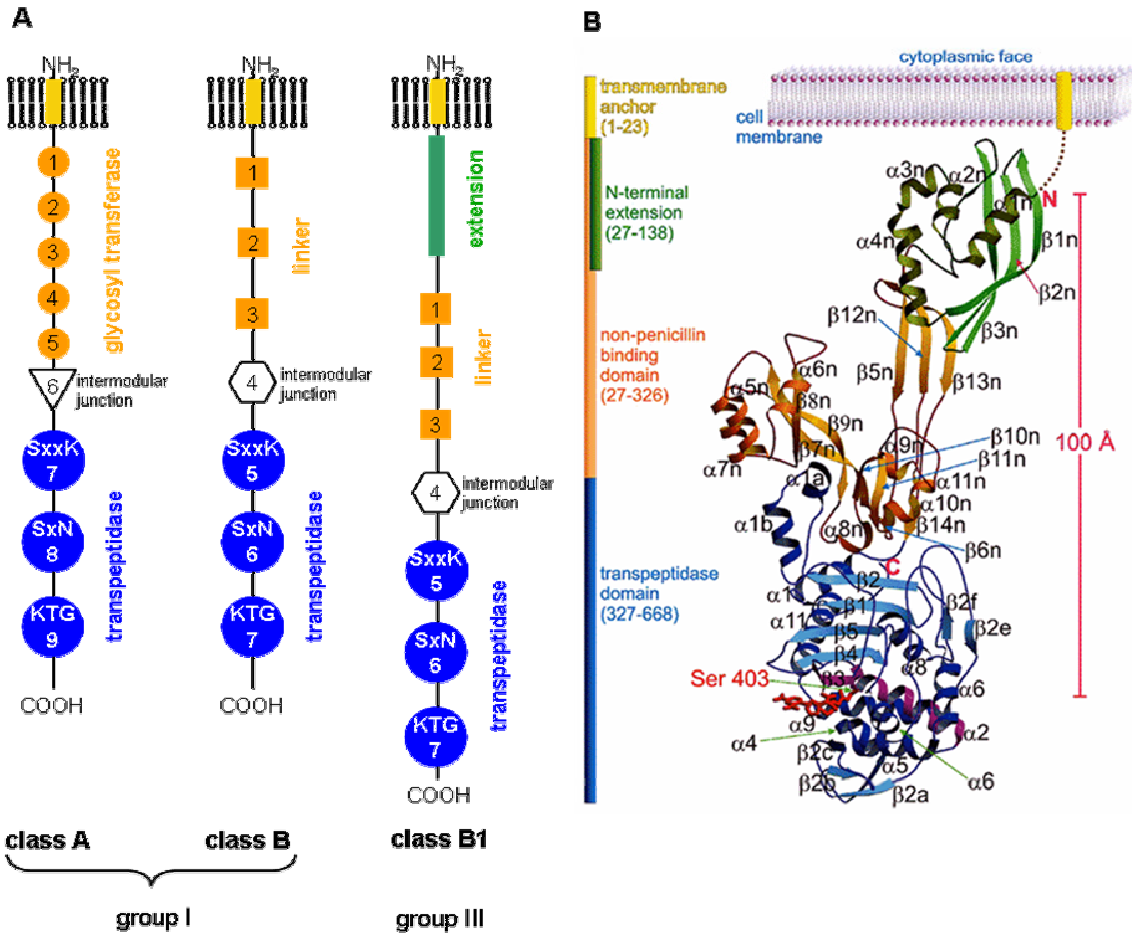


Fig. 14. Group I and III acyltransferases. **A** Distribution of class-specific motifs along the polypeptide chain. Transmembrane domain, yellow; non-penicillin binding domain, orange; intermodular junction, white; transpeptidase domain, blue. Adapted from (141). **B** Three dimensional structure of PBP2‘ (class B1, group III). The position of the active site of the transpeptidase domain is indicated by the red nitrocefin adduct (shown in stick rendering). The secondary structure elements of the transpeptidase domain were labelled in accordance with the labelling scheme used for *Streptococcus pneumoniae* R6 PBP2x (294). The N- and C-termini are labelled N and C, respectively. Shown to the left is a linear representation of the domain structure of PBP2‘ with residue numbers shown in parentheses. The colour coding is as in **A**, however without the intermolecular junction. Reproduced from (222).

A free-standing class in group I PBPs are auxiliary cell cycle proteins, implicated in cell morphogenesis. Instead of N-terminal, they have C-terminal membrane anchoring domains. The *S. aureus* transpeptidase/D-Ala$_2$ carboxypeptidase PBP4 belongs to this class and regulates the extent of peptidoglycan cross-linking (Fig. 12, A2) (106, 209). Other members have been reported to hydrolyse interpeptide linkages, allowing remodelling of the cell wall.

Group II acyltransferases are only indirectly or not implicated in cell wall synthesis,

exemplified by the β-lactamases and the β-lactam sensory transducers. For a detailed overview of the bacterial SxxK acyltransferases, the following reviews are recommended (141, 142). Contrary to other organisms, which have up to 16 PBPs (112), *S. aureus* has only four (classified in Table 1), of which PBP1 and PBP2 are essential (305).

TABLE 1. *S. aureus* acyltransferases belonging to the SxxK superfamily, classified according to (141).

Group	Class	Function	Protein	Enzymatic activity
I	A	synthesis of nascent peptidoglycan	PBP2	transglycosylase/ transpeptidase
	B	morphogenetic apparatus	PBP1	transpeptidase
			PBP3	transpeptidase
	free-standing	auxiliary cell cycle protein, here: regulation of cross-linking	PBP4	transpeptidase/ D-Ala$_2$-carboxypeptidase
II		diverse, here: indirect impact on cell wall	BlaZ	β-lactamase
			BlaR	β-lactam sensor transducer
			MecR	β-lactam sensor transducer
III	B1	β-lactam resistance	PBP2'	transpeptidase

1.3.1.2. Methicillin resistance

Upon introduction of β-lactamase resistant penicillins, like the semisynthetic oxacillin and methicillin, *S. aureus* acquired the resistance determinant *mecA*, encoding an alternative PBP; PBP2' (synonym PBP2a) (17). PBP2' is placed with other acyltransferases conferring β-lactam resistance in group III of the vast SxxK superfamily. Generally, the members of this group share to a certain extent similarity with one of the previously mentioned (sub)groups, but possess different, free-standing characteristics. For example, PBP2' belonging to the subclass B1, is similar to the class B PBPs. However, the linker region contains an essential insert, for which a structural role has been suggested in giving the transpeptidase domain a substantial reach from the cell membrane (Fig. 14). Moreover, the closed conformation of the active site of the acyltransferase module differs from the one of β-lactam sensitive PBPs, apparently hindering the acylation-reaction (141, 222). The resulting reduced acylation rate (reaction B1→B2 in Fig. 12) confers an intrinsic resistance against all β-lactam antibiotics.

Yet, upon contact with the polymeric substrate peptidoglycan, which occurs outside of the catalytic region of PBP2', a conformational change is induced, making the active site of PBP2' accessible only when the desired substrate is present and limiting the exposure to β-lactams to a minimum (119).

PBP2' is not a very efficient transpeptidase and depends on the transglycosylase nPB module of the resident PBP2 to confer resistance (77, 306). Apart from PBP2' expression, additional chromosomal factors and cell wall characteristics are required for high-level methicillin resistant *S. aureus* (MRSA), varying with the strain's genetic background (30, 331). For example, deletion of the alternative sigma factor B reduces methicillin resistance (355, 414). Borderline resistance observed in *S. aureus*, caused by overexpression of endogenous PBP2 and/or PBP4, or by mutations that reduce the penicillin affinities, lead to varying resistance levels, although always far below *mecA* linked resistance (18, 151, 160). Due to segregation of subpopulations with different resistance levels, MRSA display heterogeneous susceptibility.

The SCC*mec* element

mecA is contained in the so-called staphylococcus cassette chromosome *mec* (SCC*mec*) elements, genomic islands ranging from 21 to 67 kb (Fig. 15). These mobile genetic elements, also called resistance islands, resemble neither transposons nor bacteriophages and integrate into *orfX* with the help of type-specific cassette chromosome recombinases (*ccr*). The *ccr* gene complex is contained within SCC*mec* and is responsible for precise site- and orientation specific excision and integration. *orfX* is a gene of yet unknown function, located close to the origin of replication. Ccrs recognise the *attB*SCC site, consisting of a characteristic 15 bp direct repeat present at the 3' end of *orfX* as well as at the right end of SCC*mec*. In addition, degenerate inverted repeats characteristic of SCC*mec* are present (197). SCC*mec* is classified into allotypes by the combination of which class of *mec* gene complex and which type of *ccr* gene complex it possesses (Fig. 15). Differences in additional J regions (J for junkyard) divide SCC*mec* elements into further subtypes. Despite its disrespectful meaning, J regions can contain useful resistance genes for non-β-lactam antibiotics or heavy metals, some of which are derived from plasmids or transposons (for an overview see reference (174)).

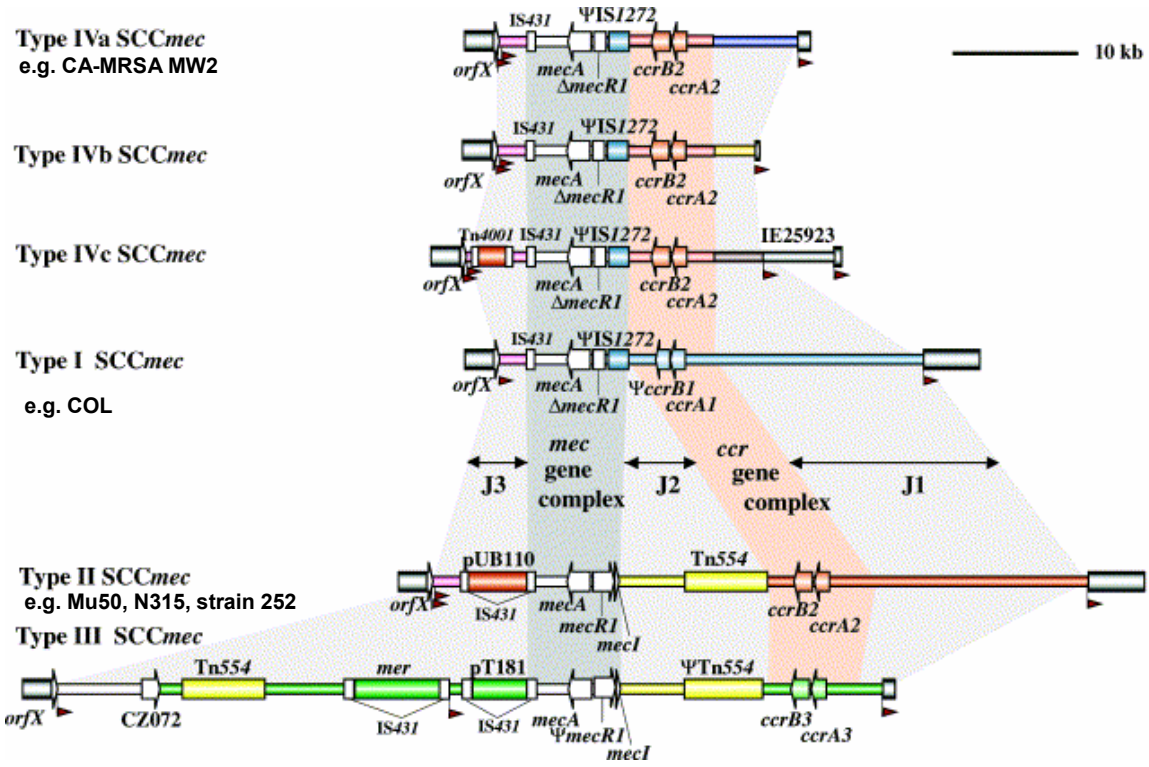


Fig. 15. Structures of SCC*mec* types. SCC*mec* is composed of two essential gene complexes, the *mec* gene complex (khaki) and the *ccr* gene complex (orange). The *mec* gene complex is responsible for β-lactam resistance and contains apart from the PBP2' encoding *mecA* two regulatory genes, *mecR1* and *mecI*. The *ccr* gene complex encodes the CcrA and CcrB recombinases. Other areas (light grey) of the SCC*mec* are non-essential, can contain various drug resistance genes or type-specific *orfs*, and are divided into three regions, J1-3. Note that in type I SCC*mec* the regulators *mecR1* and *mecI* are disrupted by the insertion sequence (IS) IS*1272* and that it contains only a pseudo (Ψ) *ccr* complex, due to a frameshift mutation in *ccrB.* In type II SCC*mec*, plasmid pUB110, mediating resistance against aminoglycosides and bleomycin, has integrated into IS4*31*. The site-specific transposon (Tn) *554*, located in J2, confers macrolide, lincosamide, streptogramin B (MLS_B) and spectinomycin resistance. Two instead of one direct repeat sequence (red arrow heads) are found in the left extremity of types II and IV SCC*mec* elements. Reproduced from (174).

Recent emergence of MRSA carriage and disease in the community has been mostly linked with type IV SCC*mec* elements (8, 231, 289), the smallest known SCC*mec*. Hospital-acquired MRSA COL, N315, Mu50 and MRSA252 and community-acquired MRSA MW2 as well as MSSA476 (13, 130, 167, 216) are the six strains whose complete sequences is nowadays published. In one of them, MSSA476, a new, non-*mecA* containing SCCmec type was found (SCC*mec*$_{476}$). It mostly resembles type IV elements, but instead of MecA it encodes a putative fusidic acid resistance protein, further highlighting the potential of these elements to act as carriers for genes that provide a selective benefit in the absence of methicillin (167).

Regulation of β-lactam resistance genes

The *mec* and *bla* operons are identically organised and expression of *mecA* is analogously regulated as *blaZ* (Fig. 16) (164).

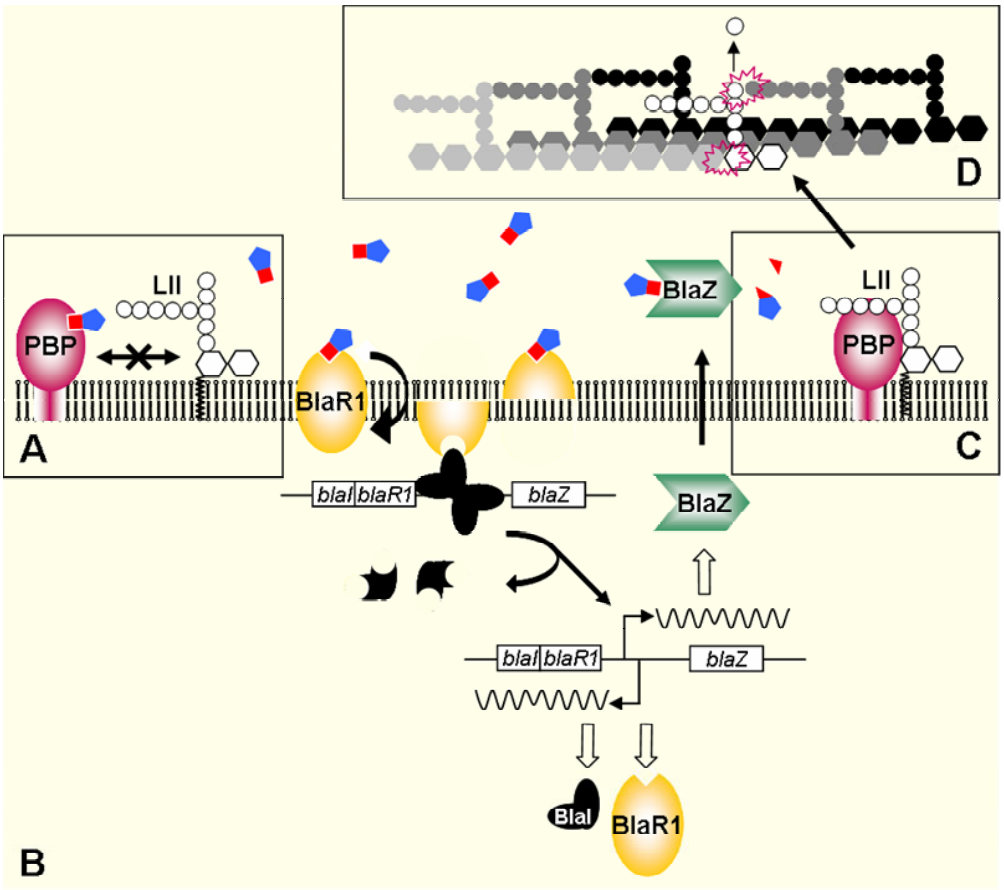


Fig. 16. β-lactam resistance mediated by β-lactamase. **A** β-lactams (red-blue particles) bind and inactivate the PBPs (purple), enzymes catalysing the incorporation of the peptidoglycan precursor lipid II (LII) into the existing cell wall. **B** The transmembrane sensor-transducer BlaR1 (ochre) senses the presence of β-lactams. Upon binding, BlaR1 undergoes autocleavage and the cytoplasmic domain, a metalloprotease, is activated. Cleavage of the cognate BlaI (black) repressor dimer follows and expression of the *bla* operon is initiated. β-lactamase BlaZ (green) is secreted and destroys the β-lactam ring (red square). **C** Thereby, PBP inhibition is abolished and transglycosylase as well as transpeptidase activity (indicated by purple explosions in **D**) of PBPs is restored, leading to the incorporation of the monomeric peptidoglycan unit of LII. Note that these two reactions involve adjacent peptidoglycan strands and that cross-linking by transpeptidase cleaves the last amino acid (D-Ala) off the stem peptide. Regulation of the *mec* operon (*mecI-mecR1-mecA*) occurs in an analogous way, the only difference being that an alternative PBP with low β-lactam affinity (*mecA)* instead of a β-lactamase is encoded. BlaZ can be secreted, as depicted here, or exist as a membrane-bound enzyme (370).

Upon induction by binding of β-lactams, the transmembrane sensors BlaR1/MecR1 proteolytically split off their cytoplasmic domain. The current model of the signal transduction mechanism is that autocleavage leaves the putative cytoplasmic proteases tethered to the membrane. Possibly through interaction with an intermediary molecule, the proteases then cleave their cognate repressors BlaI/MecI, most probably in a DNA bound state (10, 335, 420). Both repressors have been shown to bind, presumably as homodimers, *blaZ* and *mecA* DNA promoter regions and can interchangeably repress the transcription of either target gene (71, 257, 352). In addition, since the promoter regions of the divergently transcribed regulons overlap with the one of the target genes, *blaI*/*mecI* are autorepressed. In the case of a truncated regulator set, as seen for type-I SCC*mec* elements (Fig. 15), the resistance gene can be expressed constitutively, or can be regulated by the highly homologous Bla sister system, if present. Interestingly, the BlaR1/BlaI system has faster induction kinetics than MecR1/MecI (330). Although the MecR1 sensor is inducible by cefoxitin (a β-lactam), PBP2' expression is inefficiently triggered by oxacillin or methicillin, for which strains carrying only the *mec* operon can appear susceptible.

Due to the homologous regulatory region of the *mec* and *bla* operons it has been hypothesised that the *mec* determinant has evolved by recombination of a structural gene for a PBP with the regulatory region of a β-lactamase (362). The SCC*mec* element has spread *in vivo* between different staphylococcal species (407) and has entered at least on five occasions distinct *S. aureus* lineages (111). Still, MRSA predominantly disseminate clonally, suggesting that the SCC*mec* has a rather restricted mobility. Although the origin of *mecA* and the SCC*mec* element are not known, coagulase-negative staphylococci have been proposed to be the reservoir for MRSA, since they carry an identical SCC*mec* (11).

Cell wall composition

The *S. aureus* peptidoglycan comprises 30 -70% of the entire cell wall and forms a multilayered, highly cross-linked sacculus, which protects the bacterium from osmotic pressure and determines its shape (Fig. 3) (345). It consists of 20 or more layers of linear glycan chains with alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). The carboxylic group of *N*-acetylmuramic acid is substituted with the stem pentapeptide L-Ala-D-iGlu-L-Lys-D-Ala-D-Ala. A pentaglycine interpeptide, branching off the ε-amino group of the L-Lys of the stem peptide, connects one peptidoglycan chain to the D-Ala in position 4 of a neighbouring chain as depicted in Fig. 17. The flexible interpeptide allows a high, three-dimensional cross-linking of the peptidoglycan, characteristic of the staphylococcal cell wall.

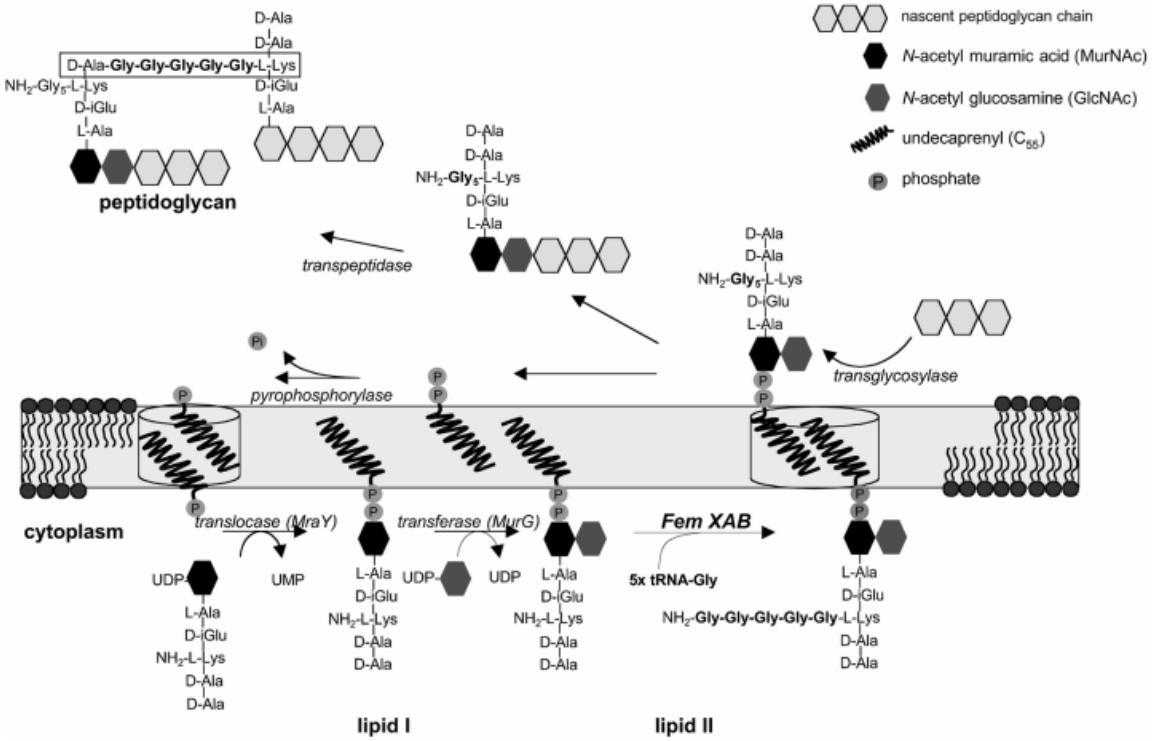


Fig. 17. Cell wall biosynthesis of *S. aureus*. Formation of the pentaglycine interpeptide bridge does not occur before synthesis of the cell wall precursor lipid II is completed. When the interpeptide bridge is accomplished, the monomeric peptidoglycan unit is translocated across the cytoplasmic membrane to the outside and incorporated into the cell wall. UMP, uridine monophosphate; UDP, uridine diphosphate. The putative flippase is symbolised by a transparent cylinder. Reproduced from (349).

Various cell wall-sorted proteins, which are important in infection and virulence of this pathogen, use the interpeptide as attachment site (302, 319). Anchoring of proteins to the cell wall is described in Fig. 18 (252). These proteins share the following features from the N- to the C-terminus: I) a signal peptide (~40 aa; SP) that directs the polypeptide into the secretory (Sec) pathway II) a segment containing a wall-spanning region, that is either rich in Pro and Gly residues or composed of Ser-Asp dipeptide repeats (e.g. the clumping factors) III) the LPXTG motif, which is recognised by the sortase A enzyme IV) a hydrophobic membrane-spanning domain serving as the sorting signal for the secretory pathway V) a series of positively charged residues at the very C-terminus remains in the cytoplasm (in Fig. 18 symbolised by a white square with a plus). For sortase B, recognising the motif NPQTN, apparently only one substrate exists: IsdC, involved in heme scavenging, is most likely tethered to the pentaglycine of already incorporated monomers and slightly buried in the cell envelope (232, 243).

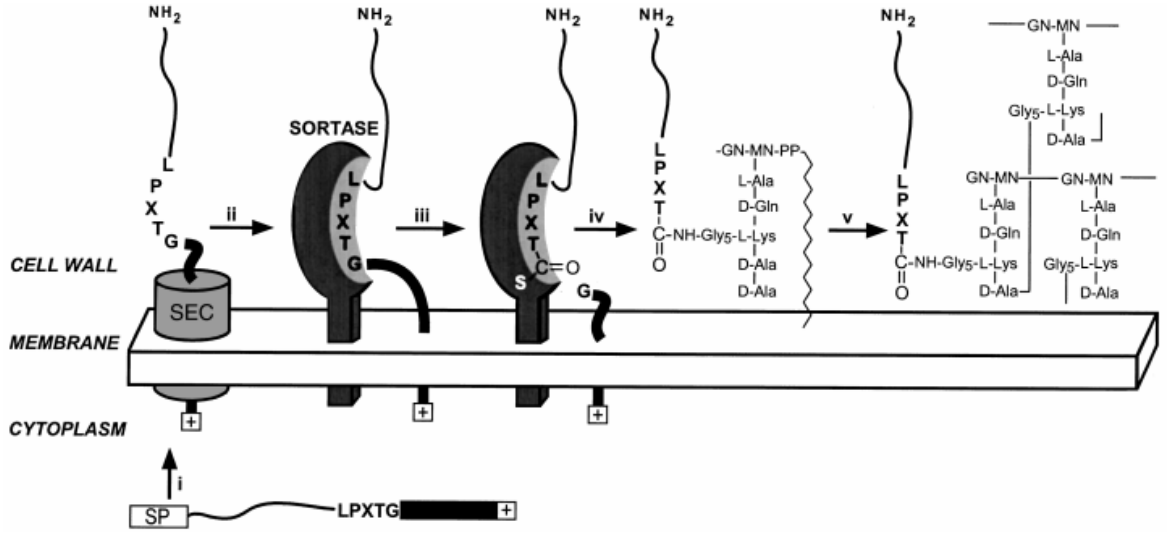


Fig. 18. Surface protein anchoring in *S. aureus*. (i) Export. Precursor proteins with an N-terminal signal peptide (SP) are initiated into the secretory (Sec) pathway, and the signal peptide is removed. (ii) Retention. The C-terminal sorting signal (black rectangular) retains polypeptides within the secretory pathway. (iii) Cleavage. Sortase cleaves between the threonine and the glycine of the LPXTG motif, resulting in the formation of a thioester enzyme intermediate. (iv) Linkage. Nucleophilic attack of the free amino group of lipid II at the thioester bond resolves the acyl-enzyme intermediate, synthesizing the amide bond between surface proteins and the pentaglycine cross-bridge and regenerating the active-site sulphydryl. (v) Cell wall incorporation. Lipid-linked surface protein is first incorporated into the cell wall via the transglycosylation reaction. The murein pentapeptide subunit with attached surface protein is then cross-linked to other cell wall peptides, generating the mature murein tetrapeptide. Reproduced from (252).

Two other possibilities for tethering wall-associated proteins are membrane attachment via a thioether-linked glyceride (274) and ionic binding via teichoic acids (84). These highly charged anionic polymers can make up 30 - 60% of the cell wall and are present in two forms; wall teichoic acids (WTA) and lipoteichoic acids (LTA). Together with peptidoglycan, teichoic acids (TA) build a meshwork providing elasticity, porosity (approximate cut off 50 kDa (168)) and tensile strength, while steering, among others, electrostatic properties and cation homeostasis of the envelope (276).

WTA consists of a linear chain of 1,3-phosphodiester-linked ribitolphosphate residues that is attached via a linkage unit to the O6 of the N-acetylmuramyl residues of the peptidoglycan (depicted below in Fig. 22). The linkage unit consists of $(Gro)_{2\text{-}3}$ManNAc-GlcNAc-1-P, where Gro stands for glycerophosphate (206). The ribitolphosphate (Rbo-P) residues are substituted with D-Ala esters and N-GlcNAc residues (Fig. 19) (403).

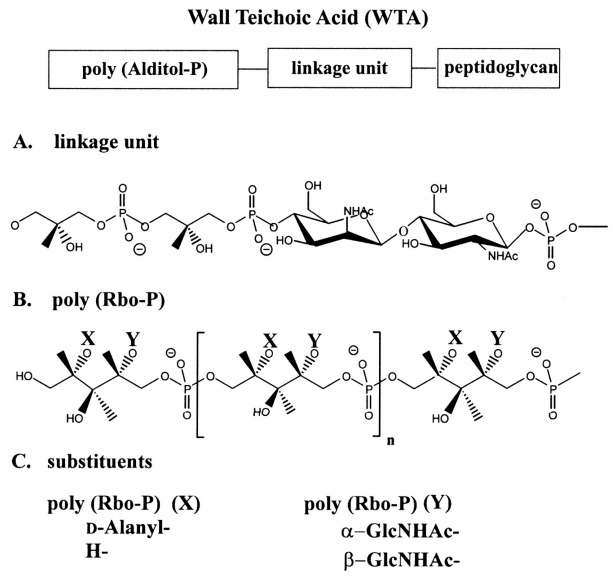


Fig. 19. Organisation of wall teichoic acid. **A** Linkage unit consisting of (glycerophosphate)$_2$-ManNAc-GlcNAc-1-P. **B** Polyribitolphosphate (Rbo-P). **C** Characteristic substituents on poly(Rbo-P). Reproduced from (276).

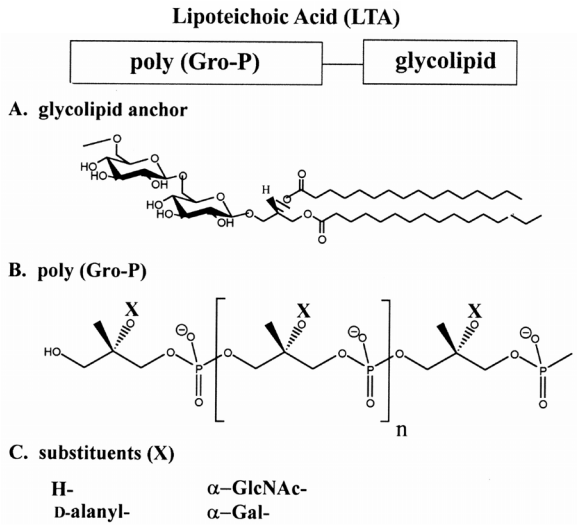


Fig. 20. Organisation of lipoteichoic acid. **A** Glycolipid anchor. **B** Poly(Gro-P) (*sn*-glycerol 1-P). **C** Substituents (X). Reproduced from (276).

LTA is build by a 1,3-linked polyGro chain that is covalently linked to the membrane glycolipid Glc(β1-6)Glc(β1-3)acyl$_2$Gro (88, 108). The Gro units are in part substituted with positively charged D-alanine esters (Fig. 20) (107, 109). While WTA and LTA have different biosynthetic pathways, the D-Ala-ester substituents of WTA are derived from those of LTA (150), into which D-Ala is incorporated by a machinery entirely encoded in the *dlt* operon (301).

Apparently, enzymatic and non-enzymatic transacylation of the chemically reactive D-Ala esters can occur, among LTA and WTA, as well as along one molecule (69, 201). Thereby, the intrinsic negative charge of these molecules is reduced by the positive charged amino group conferred by D-Ala esters (Fig. 21), a prerequisite for cell-cell approaching previous to biofilm formation (148) and adherence to host cells, possibly by causing altered binding activities of adhesins (5, 6, 250). Ala-free LTA is an inhibitor of autolysins, peptidoglycan hydrolyases required for remodelling of the peptidoglycan (110). As shown by *dlt* deletion, an increased negative net charge of the bacterium, caused by decreased D-Ala content of the teichoic acids, leads to a higher susceptibility towards innate host defence mediated by cationic peptides (72, 212, 303). On the other hand, D-Ala substituted LTA, together with peptidoglycan, is a more potent inducer of a variety of proinflammatory mediators, e.g. IL1, 6, 8 and TNF-α, than LTA alone is (Fig. 1) (200, 265, 276). The degree of teichoic acid substitution with D-Ala esters further influences susceptibility towards

antibiotics. A non-functional *dlt* operon leads to increased methicillin resistance (273), whereas a reduced D-Ala content of teichoic acids confers a higher sensitivity towards glycopeptides and lysostaphin (304).

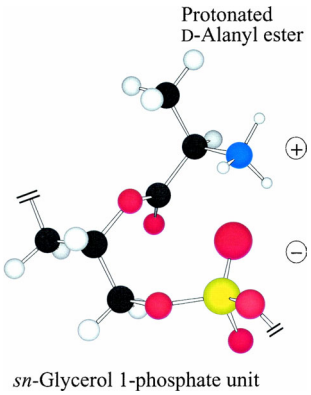


Fig. 21. Protonated D-alanyl ester substituent linked to the 2' hydroxyl of a Gro-P unit (*sn*-glycerol 1-phosphate). Ion pairing of the phosphodiester with the protonated amino group occurs on rotation of the phosphodiester linkage. Atoms are indicated by circles: hydrogen (white), carbon (black), oxygen (red), nitrogen (blue) and phosphorus (yellow). Reproduced from (276).

1.3.2. Peptidoglycan synthesis

In eubacteria, a universal biosynthesis pathway supposedly exists for the production of the UDP-MurNAc monosaccharide (387), which is further converted into the species-specific peptidoglycan precursor as depicted in Fig. 17. The stem peptide is added in a sequential fashion to the lactyl group of the MurNAc moiety by amino acid ligases (for details see ref. (55)). In some Gram-positive bacteria, the UDP-MurNAc-pentapeptide is used as substrate for the synthesis of branched peptidoglycan precursors (47, 156). The following steps are located to the membrane by MraY-mediated transfer of the soluble UDP-MurNAc-pentapeptide to the lipid carrier undecaprenylphosphate (C_{55}-P) (Fig. 17), yielding lipid I (C_{55}-P-MurNAc-pentapeptide) (162). The translocase MurG subsequently links UDP-activated *N*-acetyl-glucosamine (UDP-GlcNAc) to the muramoyl moiety of lipid I, thus yielding lipid II (C_{55}-P-GlucNAc-MurNAc-pentapeptide) (388).

Earlier experiments with membrane preparations did not unequivocally identify the substrate for interpeptide synthesis in *S. aureus*, although lipid II seemed to be preferred (193, 248). Once the synthesis of the peptidoglycan precursor is completed, it is translocated to the outside of the cell by a yet unknown mechanism. It has been suggested that the lipid anchor wraps itself around the hydrophilic glycopeptide moiety to allow crossing of the membrane (233). However, such a process is too slow for the high rate of cell wall synthesis (55, 168), given that there are not many undecaprenyl molecules (depending on the organism, 2000-10^5 per cell (260, 361, 366)) and that they have to be efficiently guided to the cytoplasmic compartment for recycling. Therefore, an enzymatically catalysed flip-flop movement is more likely. Once outside of the cell, the monomeric peptidoglycan unit is

incorporated by transglycosylation into the existing peptidoglycan and can be further used for cross-linking by transpeptidation (Fig. 12).

1.3.3. Fem factors and antibiotic resistance

The factors essential for methicillin-resistance (*fem*) of *S. aureus*, belonging to the new FemABX family of non-ribosomal peptidyltransferases (156, 324), were found by a transposon based mutation screening, identifying MRSA mutants with affected methicillin susceptibility, despite the presence of intact PBP2' (28). They were part of a group of house-keeping genes, mostly involved in cell wall biosynthesis (29, 31, 78). Extensive genetic analysis and characterization of mutant cell walls suggested that the species-specific pentaglycine interpeptide bridge is synthesised in a sequential fashion by three Fem factors. FemX, encoded by the essential gene *fmhB*, was found to be required for incorporation of the first glycine to the stem peptide (325). The second factor, FemA, was proposed to catalyse the addition of the second and third glycine (234, 367), whereas FemB is supposed to add glycines four and five (159). The Fem peptidyltransferases use $tRNA^{Gly}$ as donor in a ribosome-independent reaction (Fig. 22) (192).

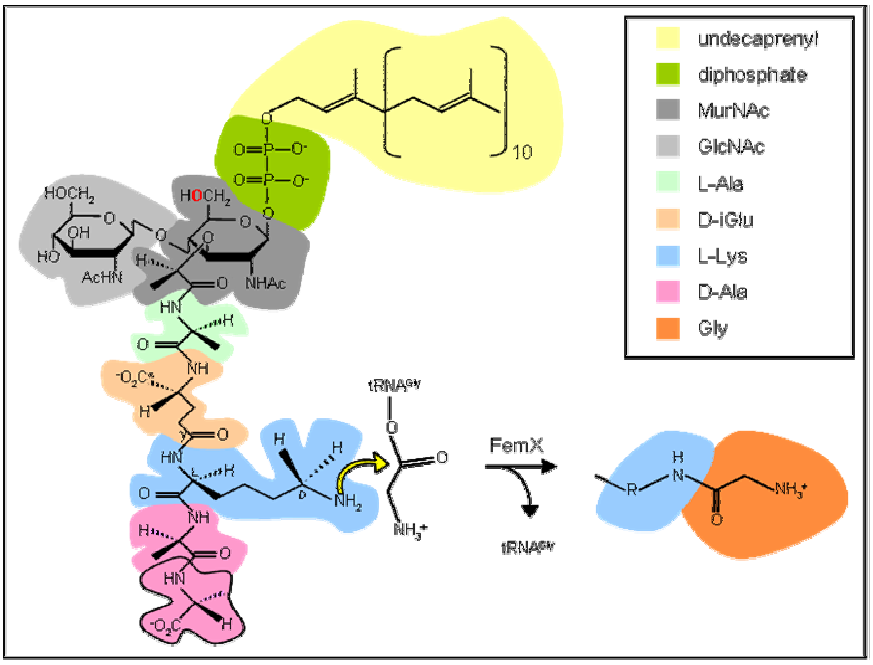


Fig. 22. *S. aureus* interpeptide bridge synthesis. Addition of the first glycine residue to L-Lys, as catalysed by the Fem factors (here FemX). In a similar way, FemA adds the next two, and FemB the last two glycine residues. Note that the direction of elongation is opposite to that which occurs during protein synthesis (and seen in the stem peptide). The nucleophilic attack of the ε-amino terminus of L-Lys on the carboxy group of the tRNAGly-linked glycine is symbolised by a yellow arrow and is not given in detail. O6 of MurNAc, the attachment site for teichoic acid, is indicated in red.

Impaired cell division, leading to aberrant septum formation and pseudomulticellular forms with thickened cell wall in *femAB* mutants further pointed at the importance of an intact pentaglycine bridge for bacterial growth (159). Moreover, *femAB* mutants become susceptible for a variety of unrelated antibiotics (224), but, conversely, more resistant against the glycyl-glycine endopeptidase lysostaphin. The defect in cell division can partially be explained by the fact that endogenous glycyl-glycine endopeptidases are involved in cell separation and that their substrate is absent in *femAB* mutants (368). Survival with a shortened bridge reduced to only one glycine requires as yet uncharacterized compensatory mutations, indicating that the interpeptide is essential for *S. aureus* (224) and required not only by PBP2', but also the endogenous PBPs. Very recently, Pinho et al. showed that recruitment of PBP2 to the division site of the cell apparently depends on substrate binding, which can also be mediated by PBP2' in the presence of β-lactams, provided proper substrate molecules are produced (305). The substrate itself has been proposed to be recruited to the division site by FtsW, a hypothetical flippase, which has been shown to localise to the division septum in an FtsZ-dependent manner in *B. subtilis* and *E. coli* (101, 229, 261). FtsZ is one of the key elements forming a ring structure at the future division site (33).

Full length interpeptide bridges seem also to be crucial for sortase; any shortening of the lipid II's pentaglycine chain leads to an impaired surface protein anchoring, and consequently affects virulence (328, 378). Homologues of FemA, FemB and FemX have been identified in all Gram-positive bacteria, which produce a branched-chain peptidoglycan with either glycine and/or L-amino acids in the interpeptide (324). Interestingly, the pneumococcal Fem homologues, MurMN and FibAB, respectively (104, 404), were found to be associated in that species with penicillin resistance as well.

1.3.3.1. Molecular structures of Fem members

Recent determination of the crystal structures of the *S. aureus* FemA ($FemA_{SA}$) alone and the *Weissella* (synonym *Lactobacillus*) *viridescens* FemX ($FemX_{WV}$) in complex with the UDP-MurNAc-pentapeptide substrate brought some light into the possible interaction modus of Fem factors and their substrates (Fig. 23). The overall globular structure of $FemX_{WV}$ is similar to $FemA_{SA}$, consisting of two almost identical subdomains separated by a cleft. Motif search revealed a certain similarity with the fold of the GCN5-related N-acetyltransferase (GNAT) superfamily, which catalyses the transfer of the acetyl group from acetyl coenzyme A to a primary amine. This superfamily comprises protein families associated with diverse

functions, including transcription factors, enzymes required for detoxification and drug resistances, metabolic enzymes and yet uncharacterised proteins. The conserved structural framework extant in all GNAT superfamily members may be modified to allow for changes in the structure of the acyl group donor, as in the case of the Fem factors, where the acyl-donor is a loaded tRNA instead of coenzyme A (393).

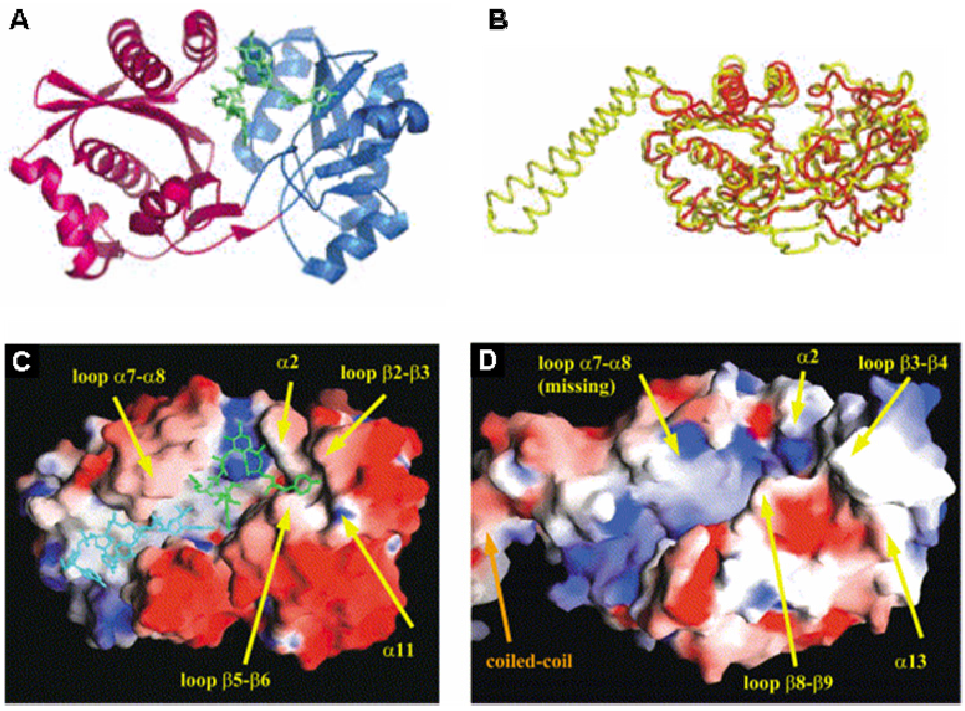


Fig. 23. Comparison of soluble and membrane-associated Fem factors. **A** The binary complex formed by *Weissella viridescens* FemX, $FemX_{WV}$, and the UDP-MurNAc-pentapeptide. $FemX_{WV}$ is constituted by two domains which are separated by a cleft: domain 1 represented in blue and domain 2 in magenta. The two domains have a similar fold. The UDP-MurNAc-pentapeptide, shown in green, lies in the cleft between the two domains and mostly contacts domain 1. **B** Ribbon plot of $FemX_{WV}$ (red) is shown optimally superimposed with *S. aureus* FemA, $FemA_{SA}$, (yellow). The overall structures are similar; the major structural difference is the absence in $FemX_{WV}$ of the coiled-coil region constituted by two helices inserted in $FemA_{SA}$ domain 2. **C**, **D** Solid surface representation coloured by electrostatic potential (blue, positive; red, negative). **C** $FemX_{WV}$ with its two substrates. The UDP-MurNAc-pentapeptide is coloured in green. The secondary structure elements of $FemX_{WV}$ domains 1 and 2 that interact with the UDP-MurNAc-pentapeptide are indicated by yellow arrows. The uracil ring is partially buried in the pocket closed by α11. The alanyl-$tRNA^{Ala}$ acceptor end, coloured in cyan, was manually docked in the domain 2 channel. **D** In $FemA_{SA}$, the loop α7- α8 was not resolved (26). The substrate specificity of members of the FemABX family, lipidic or soluble, explains the presence of the additional crevice in $FemA_{SA}$ (lipidic) formed by the loop β8-β9 and the helix α13 compared to $FemX_{WV}$ (soluble). The coiled-coil region inserted in $FemA_{SA}$ domain 2 is partially represented and is indicated by an orange arrow. Reproduced from (34).

An L-shaped channel crossing the two subdomains has been shown to accommodate in $FemX_{WV}$ the soluble acceptor, UDP-MurNAc-pentapeptide, interacting mainly with domain 1 (Fig. 23) (34). The uncomplexed $FemA_{SA}$ possesses a similar channel and an additional crevice, approximately at the site where the uracil ring of the UDP moiety is located in $FemX_{WV}$. This structural difference could account for the substrate specificity of the *S. aureus* Fem factors for lipid intermediates, lipid I or lipid II, as indicated by the inability of staphylococcal FemX to add the first glycine to UDP-MurNAc-pentapeptide (156). A further, less spectacular difference between $FemA_{SA}$ and $FemX_{WV}$ is the absence of a β hairpin. This structure lengthens the β sheet of domain 2 and strengthens the junction between the two domains. As deduced from amino acid sequence comparison, this β hairpin is also missing in $FemX_{SA}$, but present in $FemB_{SA}$ (34). Since it is only absent in the factors adding the first amino acid to the stem peptide, it could hypothetically serve the $FemA_{SA}$ and $FemB_{SA}$ factors for recognition of precursors with a substituted stem peptide.

The most striking difference between $FemX_{WV}$ and $FemA_{SA}$ was the absence in $FemX_{WV}$ of an antiparallel coiled-coil domain formed by two alpha helices. This helical arm has been proposed to provide a flexible platform for the interaction with the $tRNA^{Gly}$ substrate, as shown for $tRNA^{Ser}$ synthetase from *Thermus thermophilus* (26, 36). In addition, a 35 amino acid long region in the coiled-coil arm of the *Streptococcus pneumoniae* Fem homologue MurM has been shown to determine the preferential incorporation of alanine versus serine (105). In the case of $FemX_{WV}$, discrimination of $tRNA^{Ala}$, respectively $tRNA^{Ser}$, would therefore occur via the globular domain by a yet unknown interaction mechanism. Genome analyses of *S. aureus* revealed the presence of four or five different $tRNA^{Gly}$s, two of which have been proposed to be proteinogenic and three non-proteinogenic (57, 146), leading to the assumption that each Fem factor may use dedicated $tRNA^{Gly}$s.

The *S. aureus* FemA protein presumably contains just one $tRNA^{Gly}$ binding site, which led to the hypothesis that, after the addition of the first Gly, a subsequent round of $tRNA^{Gly}$ binding and transfer would account for the addition of the second glycine (26). Recently, direct interactions of FemA-FemA, FemB-FemB and FemA-FemB were detected using a bacterial two-hybrid system. FemX did not show any interactions with FemA or FemB. This rather suggested that FemX acts as a monomer, while FemA and FemB may act as homo- or heterodimers (323).

Staphylococcal strains producing glycyl-glycine endopeptidases, such as lysostaphin or ALE1 (369), protect their cell walls by FemABX-like immunity factors, termed Lif or Epr, which insert serine in place of glycine at positions 3 and 5 in the interpeptide (95). Interestingly, FemA and FemB were also found to interact with Lif, which was shown to substitute serine in positions 3 and 5 of the pentaglycine interpeptide chain in *S. aureus* in co-operation with FemA or FemB (95, 376). These data imply that FemA and FemB probably

function as homodimers in such a way that both subunits are loaded with $tRNA^{Gly}$ to ensure sequential addition of both Gly to the growing interpeptide bridge. Furthermore, addition of Ser at the defined positions 3 and 5 upon interaction with FemA or FemB leads to the assumption that recognition of the lipid-bound substrate is mediated by the Fem peptidyltransferases, whereas Lif may not possess an appropriate binding groove.

1.4. Small colony variants

As already mentioned above, an additional mechanism to evade antibiotic pressure and host defence is observed in persisting and relapsing *S. aureus* infections, where formation of a phenotypic mutant subpopulation occurs; the so called small colony variants (SCV) (259, 311, 350). SCVs emerge also in other nosocomial bacteria, such as *Pseudomonas aeruginosa* and *E. coli* (320, 321, 400).

Prophylactic administration of aminoglycosides (e.g. gentamicin beads as an adjunct to surgical therapy for osteomyelitis) and long-term administration of trimethoprim-sulfamethoxazole (SXT) in cystic fibrosis (CF), but not AIDS patients, strongly favours the emergence of *S. aureus* SCVs (125, 133, 188, 396, 399). Importantly, treatment with SXT might still represent a last choice for infections with MRSA, although multidrug resistant MRSA strains seem to be more frequently SXT resistant (341, 424). Apart from antibiotics, specific environments encountered in the host have been proposed to select for SCVs. For example, CF strains are more frequently hemin or menadione auxotrophic (332) and *in vitro* selection with the cationic host protein protamine leads to SCV formation (333). It has been suggested that some of the SCVs utilise an inducible and reversible resistance mechanism, avoiding fitness costs in the absence of antibiotics usually associated with resistance markers. However, the mechanism of such a switching phenotype is still unclear (246).

1.4.1. Characteristics of SCVs

Normal and SCV forms are often isolated at the same time from a patient and frequently are isogenic, as determined by PFGE (188). Therefore, diagnostic identification of SCVs requests special care, since in conventional laboratory tests the wild type phenotype masks the SCV phenotype for the following reasons:

SCVs grow slowly; typically, pinpoint colonies only appear after 48-72 h on solid medium. Their colony size is usually 1/10 of the wild type size, for which they are often overlooked. Therefore, in antibiotic susceptibility testings the presence of SCVs, which are generally more resistant towards cationic compounds like aminoglycosides and defensins,

can be missed.

Due to a reduced toxin production, hemolysis is not visible and coagulase reactions may take over 24 h to turn positive. In the case of auxotrophic SCVs, complex media may supplement the required substance(s), leading to wild type growth (259).

SCVs have an altered metabolism, for which they are not always recognised as *S. aureus* by biochemical profiling. Not surprisingly, SCVs might be erroneously identified as coagulase-negative staphylococci or contaminants (350).

Apart from these inconveniences, some SCVs even revert during the first subculture. The higher mortality of SCV-related infections compared to wild type infections (>70%, respectively 13%) (340) might be in part due to delayed or miss-identification of the highly resistant *S. aureus* subpopulation (350). More importantly, although possibly causing less damage in a short-term due to reduced toxin production, SCVs represent a constant threat since they can revert to the more virulent wild-type form, once antibiotic pressure has been removed.

Thymidine-dependent SCVs are often found in the clinical setting in correlation with long-term SXT treatment (188). The genetic reason for thymidine auxotrophy has not been studied up to date. Thymidine-dependent strains form, in addition to pinpoint, also fried-egg colonies (190). Gram-staining and electron microscopy revealed a heterogeneous morphology (Fig. 24). Cells were found to be empty or dented, covered with debris from dead bacteria and often enlarged, which had been reported before for an uncharacterised SCV (56).

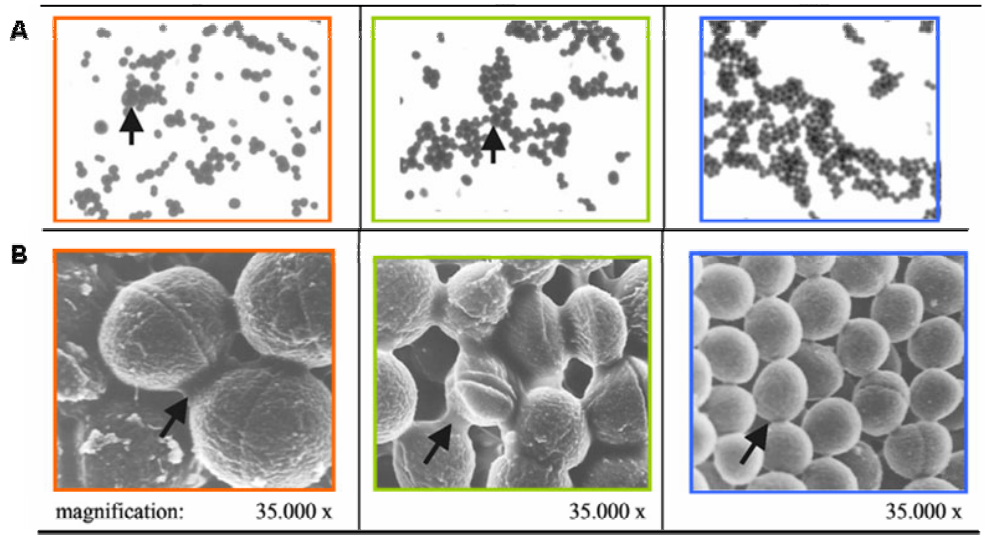


Fig. 24. Gram-staining and scanning electron microscopy of wild-type *S. aureus* (blue), pinpoint (green) or fried-egg (orange) thymidine-dependent SCVs. **A** The typical grape-like clustering of *S. aureus* was only seen with the parent strain. In SCVs pleomorphic forms were found, including colonies of sizes resembling fungi. Arrows indicate large cocci. **B** Fried-egg and pinpoint SCVs have enhanced intercellular substance compared to the wild type cells (arrows). Cells of fried-egg SCVs are in addition strongly enlarged. Reproduced from (190).

These observations could be attributed to impaired cell separation, as demonstrated by the finding of several incomplete and multiple cross walls, resulting from a reduced or undetectable splitting system (Fig. 25). An intercellular substance was more abundant compared to the normal *S. aureus* strain. The fried-egg variants reverted with a higher frequency to wild-type bacteria when cultivating without thymidine, whereas pinpoint SCVs were not able to grow without supplementation (190).

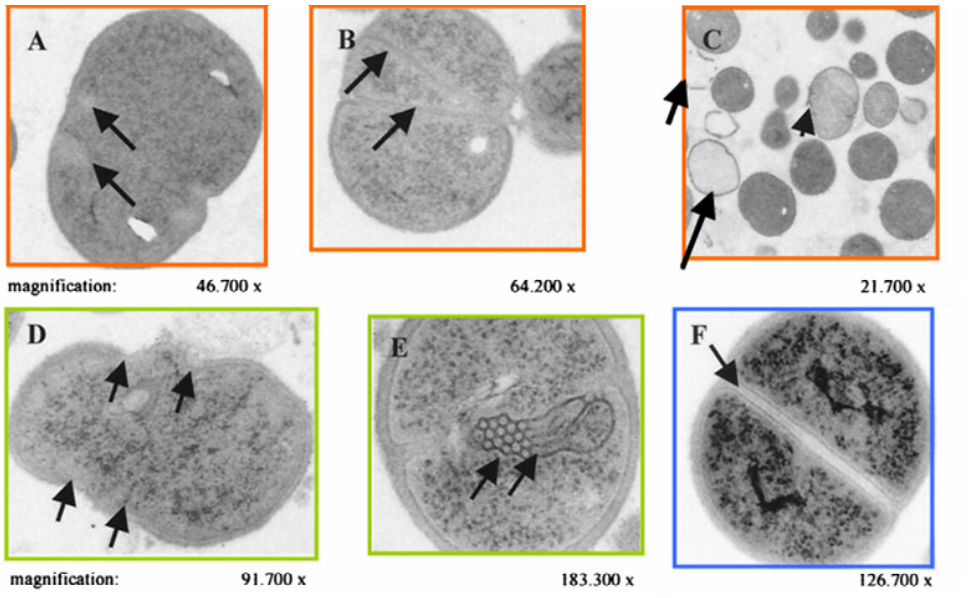


Fig. 25. Transmission electron microscopy of wild-type *S. aureus* (blue), fried-egg (orange) or pinpoint thymidine-dependent SCVs (green). Wild-type bacteria display regular cell separation by a cross wall (**F**, arrow) surrounding a highly contrasting splitting system. Fried-egg SCVs have atypical, incomplete or multiple cross walls (**A**, **B**, arrows). In **C**, besides incomplete cross walls (small arrow), cellular debris (intermediate arrow) and empty cells (large arrow) are seen. In pinpoint SCVs, atypical unrounded cells and mesosome-like structures (**E**, arrow) are found. Reproduced from (190).

Another group of clinical SCV isolates is often auxotrophic for hemin, thiamine and/or menadione (2, 311, 350), compounds involved in the synthesis of the electron carriers cytochrome and menaquinone, respectively. This observation led to the attribution of the SCV phenotype to genetic changes affecting the electron transport, although, generally, clinical SCVs may harbour additional, undefined mutations. By deleting one of the genes involved in hemin or menadione synthesis, stable mutants can be obtained that have the characteristics of SCVs (19, 20, 333, 397). A short overview of the correlation of metabolism, electron transport and virulence follows.

1.4.2. Carbohydrate metabolism and electron transport

During in vitro growth, *S. aureus* first grows on rapidly catabolisable carbon sources such as glucose, which is degraded to pyruvate (359). Per glucose molecule, 2 ATP and 2 NADH (1 NADH ≡ 3 ATP) are produced in glycolysis (Fig. 26). Pyruvate can be further converted to acetate under aerobic conditions or, in the absence of oxygen, to several fermentative products such as lactate (123, 198, 210). During the exponential phase, further degradation of pyruvate is prevented by catabolite repression of the tricarboxylic acid (TCA) cycle, producing the majority of energy from glucose in the form of 8 NADH, 2 $FADH_2$ and 2 GTP. Instead, pyruvate is converted into acetate, which is secreted during exponential growth. Upon glucose depletion in post-exponential phase, acetate is imported and directed to the TCA cycle in the form of acetyl-CoA (Fig. 26).

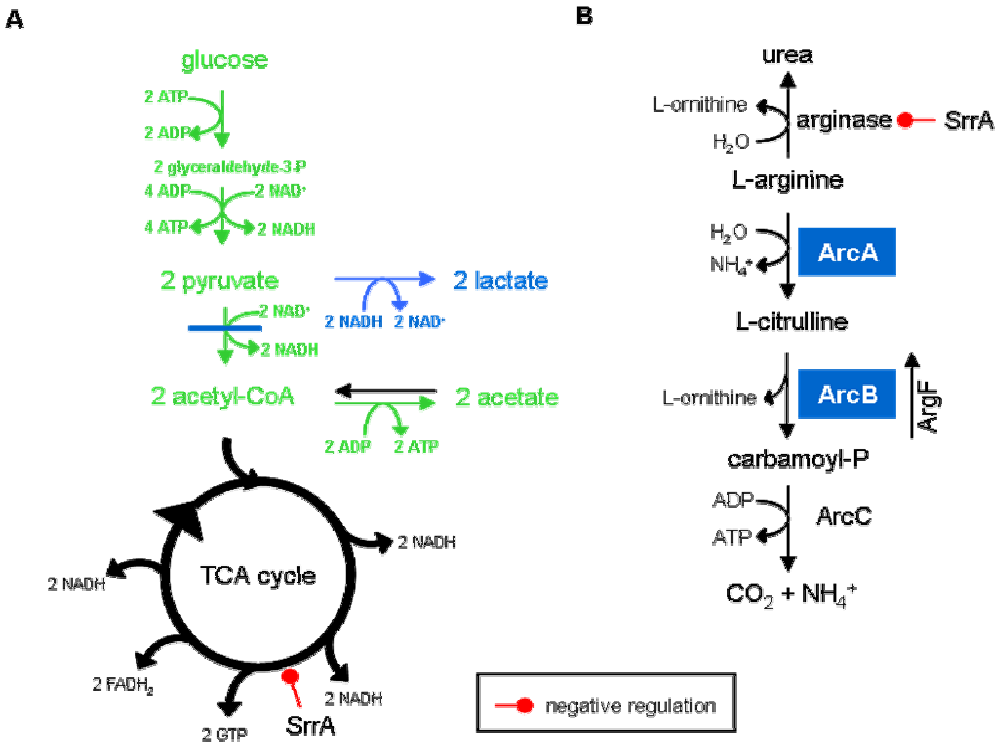


Fig. 26. Metabolic pathways in *S. aureus* under aerobic conditions. **A** Glycolysis and TCA cycle. Main reactions occurring during exponential phase (green) and in post-exponential phase (black). Blue indicates where the pathway in SCVs supposedly stops (bar) and which reactions occur instead. SrrA likely represses the TCA cycle by inhibiting the enzyme succinyl-CoA-synthase a (375). The use of glycolysis and TCA cycle yields a total of 38 ATP, while production of lactate yields only 2 ATP (1 GTP ≡ 1 ATP, 1 $FADH_2$ ≡ 2 ATP, 1 NADH ≡ 3 ATP). **B** Arginine degradation. Arginase, negatively regulated by SrrA (375), produces urea and L-ornithine. Instead, the ArcABC enzymes catalyse degradation to CO_2 and NH_4^+ by producing ATP via the arginine deiminase pathway. ArcA and ArcB have been reported to be up-regulated in a strain COL *hemB* mutant (represented by a blue box) (205).

NADH and $FADH_2$ are re-oxidised at the membrane, where a separation of electrons and protons occurs. Thereby, a proton motive force consisting of the electrical ($\Delta\Psi$) and the chemical gradient (pH) is generated. Electrons are guided back to the cytoplasm via several cell-membrane associated electron carriers with increasing redox potentials (Fig. 27). Among these are menaquinone and the cytochromes, with heme as prosthetic group. Under aerobic conditions, the final acceptor of electrons is O_2, which for the reduction to H_2O requires protons provided by the dissociation of water. This leads to an accumulation of OH^- in the cytoplasm. Driven by the energised membrane, protons from the outside of the cell flow back to the inside of the cell through the ATP synthase complex. Thereby they generate a molecular torque which is used for ATP formation from ADP and P_i (226, 233).

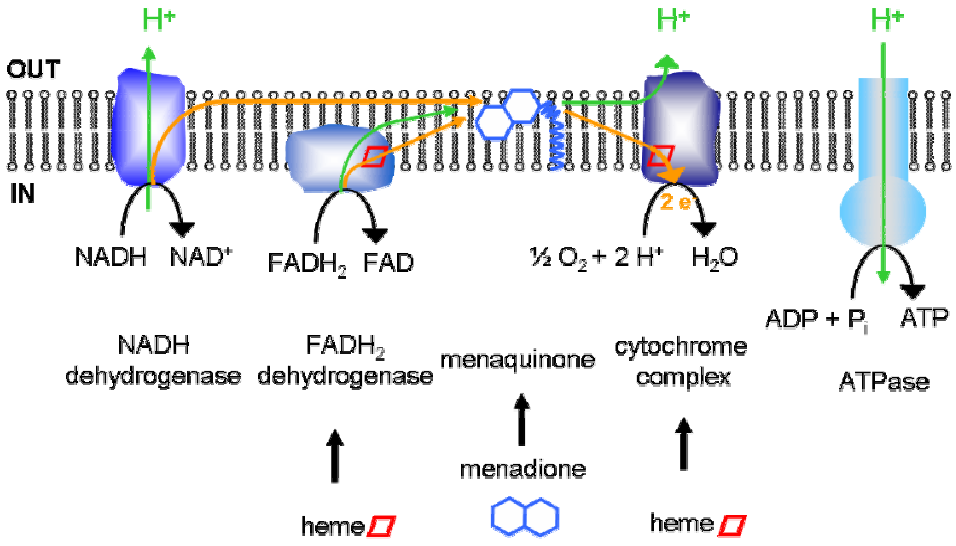


Fig. 27. Schematic representation of oxidative phosphorylation. Charge separation of protons (green arrows) and electrons (orange arrows) from the reduced equivalents NADH and $FADH_2$ occurs by the corresponding dehydrogenases. While protons accumulate on the outside of the cells, electrons are guided back to the cytoplasm via several carriers, among which menaquinone and cytochromes. Thereby, an electrical ($\Delta\Psi$) and a chemical (ΔpH) gradient builds up, which together provide the proton motive force driving ATP production by ATPase. Heme is represented by a red rhombus, menadione by two blue, fused hexagones. Isoprenylation, which yields menadione, is depicted with a blue wave line.

An interrupted electron transport chain, as found in hemin, thiamine and/or menadione auxotrophic SCVs, leads to the accumulation of reducing equivalents. Metabolic pathways depending on the availability of the oxidising equivalents $NAD(P)^+$ and FAD, like the TCA cycle, are inhibited, which drastically reduces the range of carbohydrate utilisation as well as energy production (317). The block in electron transport chain forces SCVs to reoxidize NADH via different pathways. Apparently, this happens through increased fermentative activity, as reflected by up-regulated lactate and alcohol dehydrogenase

production in a COL *hemB* mutant. As expected, TCA cycle enzymes are down-regulated in such a mutant, manifesting a divergent metabolic activity compared to wild type *S. aureus* (205). Obstruction of the electron flow furthermore hinders charge separation, leading to a low membrane potential and reduced ATP production (220, 310). Consequently, ATP-requiring biosynthesis of proteins, cell-wall or nucleotides is limited and finally cells stall their growth. Reduction in cellular ATP levels indirectly blocks the first step of the phosphotransferase system, inhibiting the use of sugars such as mannitol, xylose, lactose, sucrose, maltose and glycerol (317). Processes requiring electron transport, such as pigment formation or synthesis of phosphatidyl glycerol, the precursor of teichoic acid, are affected as well (143, 186, 195). However, the reduced trans-membrane potential protects bacteria from cationic antibiotics (20), slow growth hampers the efficacy of substances targeting metabolic active cells and the low toxin production allows them to persist in the host cell, a relatively protected environment (16).

1.4.3. Expression of global regulators and virulence determinants

Enzymes involved in the ATP-producing arginine dihydrolase pathway are increased in a COL *hemB* mutant (205), suggesting the alternative arginine degradation by arginase, which does not generate ATP, being inhibited in SCVs. Lactate and alcohol dehydrogenase, as well as arginase, are proteins influenced by SrrA (375), for which an involvement of this two-component system in *hemB* mutants seems probable (Fig. 26). Another regulator possibly involved in the control of metabolism is SigB, as it has been shown to be required for acetate degradation (360). The second enzyme of the highly conserved TCA cycle, the aconitase CitB, was found in *B. subtilis* and *E. coli* to bind in an iron-dependent fashion to mRNA-elements, as reported for eukaryotic aconitases (196), and therefore could be a candidate for post-transcriptional regulation. A non-functional TCA cycle leads to the reduced production of several virulence factors, such as toxins and lipase (359).

Previous analyses of strains 8325-4 and Newman *hemB* mutants had revealed that protein A, α-toxin and coagulase are only weakly produced (184, 397). In contrast, the adhesion proteins FnbA and FnbB, as well as the fibrinogen-binding ClfA are up-regulated (391). The mRNAs from the major regulatory locus *agr* are differentially expressed. While RNAII levels are apparently not influenced, RNAIII levels are dramatically reduced in a *hemB* mutant with 8325-4 background (391). In parallel to *agr*, the overall *sar* mRNA levels are decreased in the *hemB* mutant compared to the parent. However, due to the applied RT-PCR technique, no distinction between the three overlapping transcripts originating from the *sar* locus is made in this study.

In the before mentioned COL *hemB* mutant, increased amounts of the

immunodominant antigen A are found in stationary phase (205). This extracellular protein has been shown to be directly regulated by YycF, although it has not been reported whether positively or negatively (86).

These observations support the idea that the extraordinary physiologic state of SCVs is likely linked to an altered activity of global regulators and expression of virulence factors, *in vitro* as well as *in vivo*.

1.5. Aims of this work

I Cell-membrane associated steps of peptidoglycan synthesis

For further analysing the individual steps of interpeptide formation in *S. aureus* and to identify the correct substrates, the usage of undefined membrane preparations will be bypassed by purifying lipid I and lipid II, the essential enzymes FemA, FemB, FemX, the $tRNA^{Gly}$ synthetase and total tRNA. The thereby reconstituted *in vitro* assay would allow the study of substrate-enzyme and protein-protein interactions necessary in the membrane-bound steps of staphylococcal cell wall synthesis.

II Temporal patterns of global regulators in a *hemB* mutant

In this study, various global regulators and their effect on selected virulence determinants were monitored in a Newman derivative and its *hemB* mutant. The use of one defined genetic background would eliminate strain dependent variations. Analyses under the same experimental conditions would provide for the first time a compilation of temporal transcription patterns, contributing to the elucidation of the regulator's interplay during growth.

2 RESULTS I

2.1. Setup of the assays and detection system

Membrane preparations possess enzymatic activity (MraY and MurG) for the formation of cell wall precursors (389); membranes of *S. aureus* have been reported to contain Fem protein activities and to synthesize glycine-labelled lipid intermediates when complemented with soluble cellular fraction (248). As shown with *Staphylococcus simulans* 22 membranes (Fig. 28), the lipid carrier molecule (C_{55}-P) remained unmodified in the absence of UDP-activated precursors. Upon addition of UDP-MurNAc-PP, C_{55}-P was transferred into lipid I; the conversion was incomplete because of the reversibility of the MraY-catalysed reaction (308). When UDP-GlcNAc was also present, most of the lipid I was converted to lipid II, which migrates somewhat more slowly.

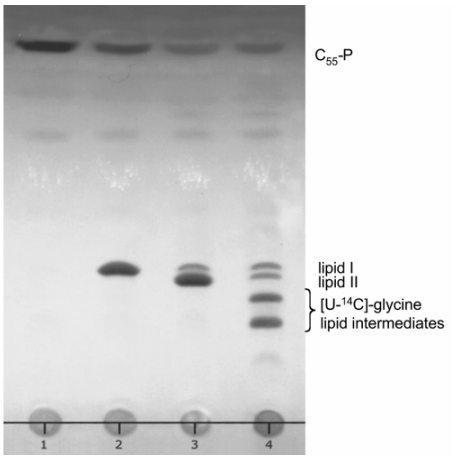


Fig. 28. Thin layer chromatography (TLC) of [U-^{14}C]-glycine-labelled lipid intermediates. Undecaprenylphosphate (10 nmol; C_{55}-P) was incubated *in vitro* with membranes (360 µg of protein) of *S. simulans* 22 for 60 min in the presence of 50 nmol of glycine lacking the UDP-activated precursors (lane 1). Lipid I was synthesised upon addition of UDP-MurNAc-PP (lane 2) and lipid II by addition of UDP-MurNAc-PP and UDP-GlcNAc (lane 3). Two glycine-labelled lipid intermediates migrating more slowly than lipid I and lipid II were separated after incubation with UDP-MurNAc-PP and UDP-GlcNAc and addition of supernatant I (330 µg of protein) (lane 4). Reproduced from (349).

Using lipid II from a preparative batch, its position on the chromatogram was verified, and its molecular mass was confirmed by mass spectroscopy (Fig. 29.1).

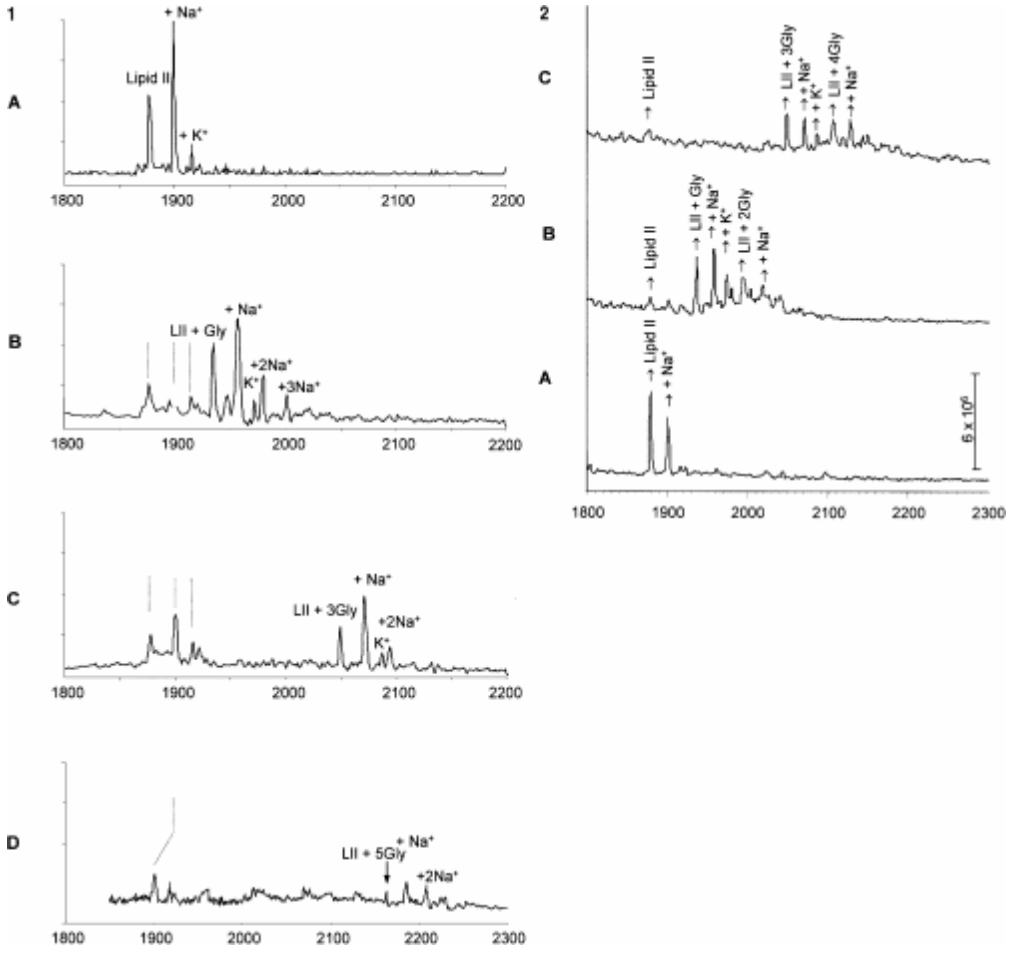


Fig. 29. 1 ES-MS spectra were obtained with an API-I single quadrupole instrument (PE-SCIEX) running in a positive mode. Peaks at *m/z* 1878 correspond to lipid II (**A**), at *m/z* 1935 to lipid II-Gly_1 (**B**), at *m/z* 2049 to lipid II-Gly_3 (**C**) and at *m/z* 2163 to lipid II-Gly_5 (**D**). Other peaks correspond to the non-covalent sodium or potassium ion adducts. **2** With prolonged incubation time (90 min) and elevated enzyme concentrations (molar ratio of enzyme:lipid II, 1:50), lipid II-Gly_2 (**B**) at *m/z* 1992 and lipid II-Gly_4 (**C**) at *m/z* 2106 were detected in addition to the correct products. Reproduced from (349).

When radiolabelled glycine was added to the assay, no further bands were observed (Fig. 28), and both bands representing lipid I and lipid II did not contain radiolabel. In contrast, when the assay mixture was supplemented with the soluble cytosolic fraction obtained after low-speed centrifugation (supernatant I), two additional bands occurred with R_f-values lower than that of lipid II (Fig. 28, lane 4). Both bands were radiolabelled, strongly suggesting that they represent lipid I/lipid II intermediates with different segments of the glycine interpeptide bridge attached. Moreover, from the intensities of the various bands obtained after phosphomolybdic acid (PMA) staining, it appeared that lipid II was the major substrate for the Fem-catalysed reaction, as it was strongly reduced on the chromatogram in favour of the two newly formed glycine-containing intermediates.

2.2. *In vitro* activity of FemX, FemA and FemB

Although the above system, based on the use of soluble cytosolic fractions, was sufficient to determine that all factors necessary for interpeptide formation (i.e. the Fem factors, glycyl-tRNA-synthetase and tRNA) were present, it did not allow for a detailed step-by-step analysis of bridge formation. In particular, the presence of MurG activity in the supernatant prevented unequivocal determination of whether lipid I or lipid II was the substrate for FemX. High-speed ultracentrifugation (supernatant II) clearly reduced MurG activity; however, the overall reaction was strongly reduced, presumably because of simultaneously removing the Fem enzymes and/or glycyl-tRNA-synthetase and tRNAs (data not shown).

Therefore, FemX, FemA, FemB and the $tRNA^{Gly}$-synthetase (GlyS) of *S. aureus* strain 8325 were His-tagged and purified (Fig. 30).

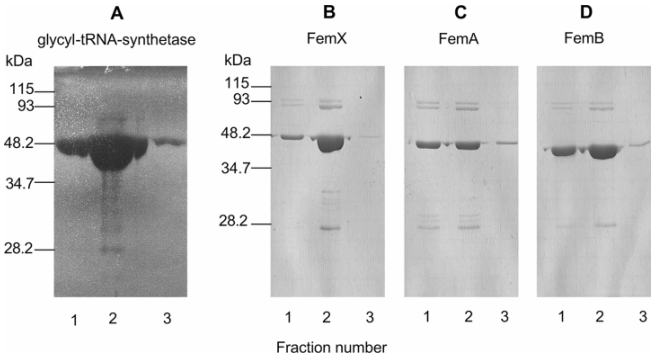


Fig. 30. 10% SDS PAGE of recombinant proteins. His-tagged enzymes were purified on a Ni-NTA column. Three 500 µl fractions were collected, to which an equal amount of 100% glycerol was added for preservation at -20 °C. GlyS fractions (10 µl) and 1 µl of FemABX fractions were loaded onto the gel. Prestained broad-range standard marker from Bio-Rad was used to estimate molecular masses. Fractions 1-3 of GlyS (**A**). Fractions 1-3 of FemX (**B**). Fractions 1-3 of FemA (**C**). Fractions 1-3 of FemB (**D**). The following fractions were selected for further experiments: A2 (GlyS); B2 (FemX); C1 and C2 (FemA); D1 and D2 (FemB). Reproduced from (349).

In addition, total tRNAs were isolated from both *S. simulans* 22 and *S. aureus*. Replacing the supernatant by recombinant FemX, FemA and FemB, GlyS and purified tRNAs resulted in the formation of glycine-labelled lipid intermediates (Fig. 31).

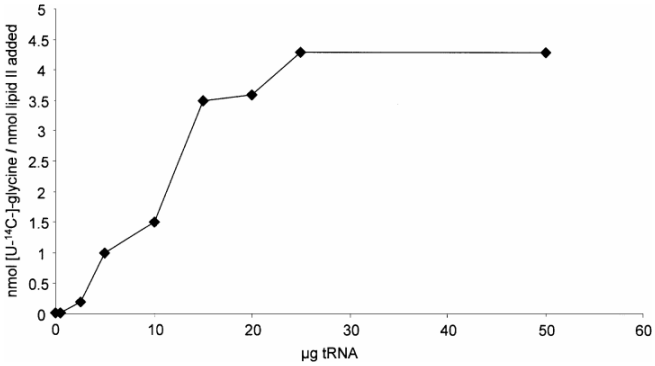


Fig. 31. Influence of increasing tRNA concentrations on the FemXAB reaction. Purified lipid II was incubated with [U-^{14}C]-glycine in the presence of FemXAB and recombinant tRNA synthetase with increasing concentrations of purified tRNAs. Reaction products were separated by TLC, and incorporation of [U-^{14}C]-glycine was determined by scintillation counting. Reproduced from (349).

Under the conditions chosen, i.e. with an excess of [U-^{14}C]-glycine and a defined amount of 5 nmol of lipid II in the system, the availability of dedicated glycyl-tRNA was rate limiting. At a concentration of 25 µg of total tRNA per assay volume, the maximum amount of pentaglycine bridge formation was obtained. There was no difference observed using purified tRNAs from *S. simulans* 22 or *S. aureus*. Assuming that all lipid II molecules were available, a 5:1 ratio of glycine to lipid II was expected in the final product. As indicated in Fig. 31, the maximum amount of glycine incorporation obtained in the *in vitro* system corresponded to an average of 4.3 mol of glycine incorporated per mol of lipid II.

2.3. The cell wall precursor lipid II is the only substrate of FemX

Identification of the substrates of the FemX- and subsequently of the FemA- and FemB-catalysed reactions was achieved by incubation of lipid I or lipid II (Fig. 32) in the presence of GlyS and tRNAs, with FemX, FemX and FemA and FemX, FemA and FemB. Analysis of the reaction products by thin-layer chromatography (TLC) after incubation of lipid I with the Fem proteins identified lipid I as a poor substrate for FemX, and no further incorporation of glycine was detected after incubation with additional FemA and FemB (Fig. 32B).

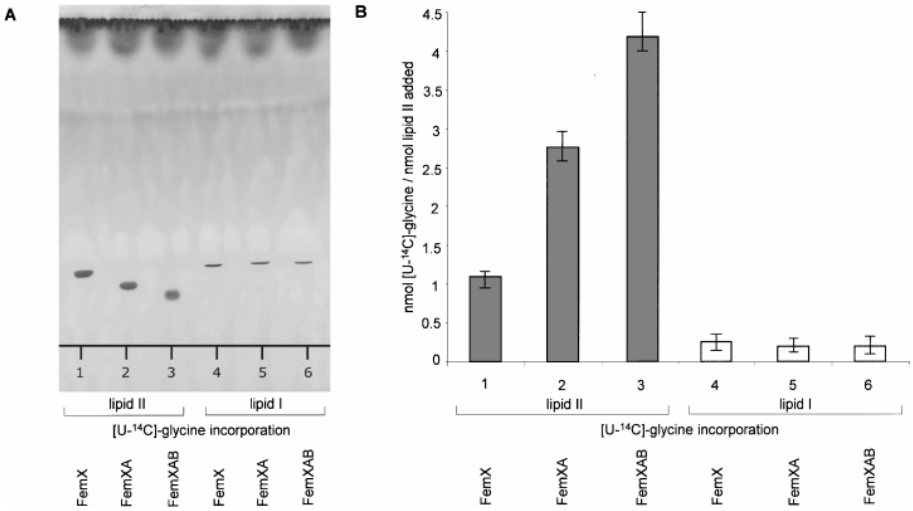


Fig. 32. A TLC of glycine-labelled lipid intermediates synthesized by recombinant FemX, FemA and FemB. Purified lipid II (lanes 1-3) and lipid I (lanes 4-6) were incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA-synthetase and purified tRNA with FemX (lanes 1 and 4), FemX and FemA (lanes 2 and lane 5) and FemX, FemA and FemB (lanes 3 and 6). **B** [U-^{14}C]-glycine:lipid ratio of the analysed glycine lipid intermediates. Mean values of five experiments are given. Reproduced from (349).

In contrast, incubation of lipid II resulted in the formation of glycine lipid II intermediates migrating stepwise more slowly in the TLC system upon addition of glycine to the pentapeptide side-chain of lipid II (Fig. 32A). The molar ratio of radiolabelled glycine to lipid intermediate indicated that FemX (glycine:lipid II, 1.3:1) added the first glycine to lipid II. The glycine:lipid incorporation ratios for the FemXA and the FemABX reactions were 2.76:1 and 4.17:1 as expected after the formation of lipid II-Gly_3 and -Gly_5 (Fig. 32B). Lipid II itself, i.e. without the first glycine added by FemX, was not used by FemA and Fem B, either on their own or combined. Only background Gly incorporation levels, in the range of

0.02-0.2 Gly incorporated per lipid II molecule, were observed. Although the incorporation levels were somehow lower than expected theoretically, especially for the FemABX reaction, analysis of the products by mass spectrometry confirmed the formation of lipid II-Gly_1 by FemX, lipid II-Gly_3 by FemXA and lipid II-Gly_5 by FemXAB (Fig. 29.1). It should be noted that determination of glycine incorporation ratios required, first, extraction of radiolabelled intermediates from the synthesis assay using n-butanol/pyridine-acetate, subsequent TLC and, finally, counting of excised bands; this procedure is likely to cause some loss of radiolabelled products and, obviously, the extraction yields decrease with increasing bridge length of the products.

Lipid II with intermediate glycine contents (lipid II-Gly_2 and lipid II-Gly_4) was not detected under these conditions (Fig. 29.1). However, when the assay conditions were changed (90' incubation and a lipid II:enzymes ratio of 50:1), such products were detectable (Fig. 29.2). Under the experimental conditions chosen, i.e. lipid II in micellar form and in the presence of 0.8% Triton X-100, FemX not only accepts lipid II as a substrate but also lipid II-Gly_1. Accordingly, after 90 ' of incubation, the reaction products of FemXA contained lipid II-Gly_3 and lipid II-Gly_4 (Fig. 29.2). Obviously, FemA can also accept lipid II-Gly_2 as a substrate and then incorporate two additional glycine residues.

As reported previously, UDP-MurNAc-PP is the substrate for the soluble recombinant FemX proteins of *Weissella viridescens* and *Enterococcus faecalis* (48, 156). To exclude UDP-MurNAc-PP as a substrate for the Fem proteins of *S. aureus*, 5 nmol of lipid II was incubated with FemXAB, glycyl-tRNA-synthetase and tRNAs in the presence of 5-100 nmol of purified UDP-MurNAc-PP isolated from *S. simulans* 22. Analysis of the reaction products revealed that the addition of glycine to lipid II was not affected even in the presence of a 20-fold molar excess of UDP-MurNAc-PP. This competition experiment demonstrated that the FemXAB enzymes of *S. aureus* catalyse the addition of glycine to the pentapeptide side-chain only after the formation of lipid II. This result is in good agreement with the fact that UDP-MurNAc hexapeptides were detectable in the cytoplasm of *W. viridescens* (307), but not in *S. aureus* (364) or in the staphylococcal strains used in this study (data not shown).

2.4. Time course of the FemXAB reactions

The formation of the Gly-containing lipid II intermediates was followed over time (Fig. 33). Initial Gly addition by FemX was already completed after 5' and resulted in a glycine:lipid II ratio of 1.22:1(see above). In contrast, the presence of FemA in the synthesis assay decreased the formation of lipid II-Gly_1 reaction products, indicating that, in our *in vitro* system, the presence of FemA interferes with Gly addition by FemX. The maximum level of the FemXA reaction was reached after an incubation period of 60' with a final glycine:lipid II ratio of 3:1. An even stronger effect was observed upon addition of FemB. The FemX reaction was retarded further, and glycine lipid II intermediates were not detected before 15' of incubation. The synthesis was not completed within 90' of incubation.

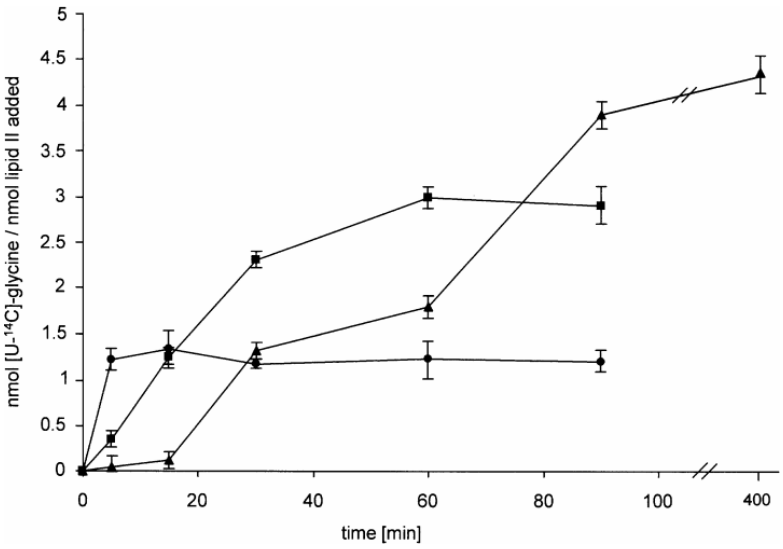


Fig. 33. Time dependency of the FemXAB reactions. Purified lipid II was incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA synthetase and tRNA with addition of FemX (circles), FemX and FemA (squares) and FemX, FemA and FemB (triangles); mean values of three experiments are given. Reproduced from (349).

2.5. Analysis of the individual Fem protein reactions

As the maximum catalytic activity of FemX was observed in the absence of FemA and FemB (Fig. 33), the unlabelled FemX and FemA reaction products were purified to study the FemA and FemB reactions independently (Fig. 34). Using the standard assay, comparable amounts of labelled Gly per lipid were added to lipid II-Gly_1 by FemA alone (1.73:1) and by FemA when FemX was present (1.90:1). Again, the observed incorporation was somewhat lower than the theoretically expected value of two Gly per lipid II-Gly_1 incorporated, as discussed above. This result clearly demonstrates that FemA activity *in vitro* does not depend on the presence of FemX. Neither FemX nor FemB catalysed further addition of glycine to lipid II-Gly_1, proving the preference of FemX for lipid II and of FemB for lipid II intermediates containing at least three Gly.

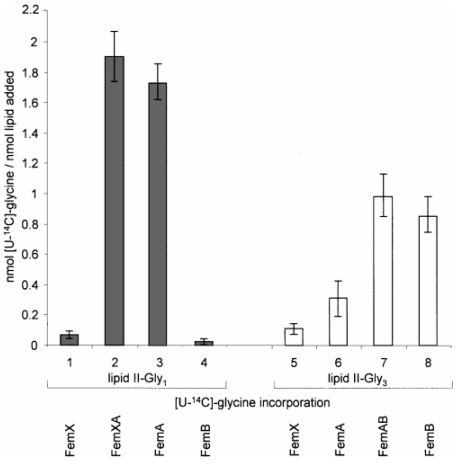


Fig. 34. Analysis of the FemA and FemB reaction using purified lipid II-Gly_1 (grey columns) and lipid II-Gly_3 (white columns). Purified FemX product (grey) and FemXA product (white) were incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA-synthetase and purified tRNA with FemX (columns 1 and 5), FemX and FemA (column 2), FemA only (columns 3 and 6), FemA and FemB (column 7) and FemB only (columns 4 and 8); mean values of two experiments are given. Reproduced from (349).

The overall incorporated radioactivity by FemB and FemAB suggested that only one more Gly residue may have been added to the lipid II-Gly_3 (Fig. 34); however, mass spectroscopy clearly showed that only lipid II-Gly_5 occurred as a product (Fig. 29.1). This further indicates that extraction yields from the synthesis assay decrease strongly with interpeptide chain length (see also above).

3 DISCUSSION I

Earlier studies on peptidoglycan precursor synthesis used crude extracts of the membrane and cytoplasmic compartment which were further fractionated. Thereby, it could be shown that *S. aureus* uses mainly lipid II as acceptor for pentaglycine bridge formation and that only part of the $tRNA^{Gly}$s could be used as donor for pentaglycine bridge formation (193, 248, 249). However, due to the presence of contaminating molecules or suboptimal reaction conditions, these assays did not clearly identify the substrates for pentaglycine addition. Furthermore, the proposed biochemical function of each Fem factor had not been tested *in vitro*, leaving the question of whether multienzyme-complex formation or additional proteins were required for activity unanswered.

The established *in vitro* system using purified bactoprenol-bound and soluble cell wall precursors as well as His-tagged enzymes allowed detailed analysis of single steps of glycine addition. Together with purified staphylococcal tRNAs and GlyS from *S. aureus*, this synthesis assay constituted an entirely homologous and defined system. In contrast to earlier studies (192), glycine incorporation using tRNA-synthetase and tRNAs isolated from *Escherichia coli* when used in combination with *S. aureus* tRNAs or tRNA-synthetase, respectively, could not be observed (data not shown). Neither the soluble UDP-MurNAc-pentapeptide, nor lipid I, was converted to glycine-labelled precursors by the FemABX peptidyltransferases.

Since lipid II was therefore the only substrate of the Fem factors, analyses of the single steps of glycine incorporation by the Fem proteins was performed only with lipid II. The substrate was completely converted to lipid II-Gly_1 by FemX within 5', which corresponds to a turnover rate of 19 lipid II molecules per FemX molecule $minute^{-1}$. The ratio of FemX:lipid II in the *in vitro* assay was ≈1:100. Such a turnover rate is in the same order of magnitude as that calculated recently for *E. coli* K-12. 24 disaccharide peptide units need to be transported per minute per bactoprenol carrier ($3.5 \cdot 10^6$ disaccharide peptide units per cell; generation time 36'; 50% turnover per generation; about 2000 lipid II molecules per cell) (260). However, it is not known whether the turnover of lipid II in Gram-negative and Gram-positive bacteria is similar, since the amount of peptidoglycan per dry weight differs greatly, with about 10% in Gram-negative and about 30-70% in Gram-positive bacteria. Still, when compared with *E. coli*, the number of lipid II molecules in staphylococci is 25 times higher than that determined for *E. coli* (361), leading to the assumption that synthesis of the cell wall in Gram-positive bacteria depends on the amount of lipid II rather than on an increased synthesis rate.

A rate-limiting factor for the FemABX reaction *in vitro* was the availability of glycine donor tRNAs. Although increasing concentrations of tRNAs lead to increasing amounts of incorporated glycine, the FemABX reaction never reached the expected values of Gly_5. Similarly, amino acid analysis of peptidoglycan synthesized *in vivo* revealed submaximal values of incorporated glycine, reflecting a minor presence of Gly_3 and Gly_1 interpeptide bridges (95, 326, 367, 380). However, as confirmed by mass spectrometry, the only *in vitro* FemX product is lipid II-Gly_1, and FemXA only produced lipid II-Gly_3, and no lipid II-Gly_2 (Fig. 29.1). This observation agrees with the hypothesis that FemAB add two glycine molecules per cycle, not releasing a lipid II-Gly_2, respectively lipid II-Gly_4, product, whereas FemX clearly adds only one Gly per cycle to the lipid intermediates. The molecular basis for such a difference in the catalytic mechanism has not yet been resolved. Benson et al. proposed that the 12 amino acids of FemA, which had not been resolved by X-ray analyses, could close down over the channel during the addition of each glycine and then reopen for release of the product (26). Another possibility could be a quick and save handing-over of the intermediates lipid II-Gly_2, respectively lipid II-Gly_4 from one FemA/B factor to the other FemA/B factor, probably by homodimerisation.

The fact that only lipid II and its Gly additives were substrates of the Fem factors (Fig. 32), suggested the Fem factors to discriminate GlcNAc-MurNAc versus MurNAc lipid intermediates. Whether this function is accomplished in the L-shaped groove found in FemA remains to be determined; as judged by the size, an accommodation of the disaccharide seems to be possible (Fig. 23). Competitive, non-productive binding of lipid II by recognition of the disaccharide unit alone, regardless of the amount of Gly added, could explain the stepwise reduced incorporation rates in the presence of further Fem factors (Fig. 33). Correspondingly, the non-productive binding by FemA and FemB would delay lipid II-Gly formation, FemX and FemB the one of lipid II-Gly_3, and binding by FemX and FemA the production of lipid II-Gly_5. Still, the appropriate number of Gly in the interpeptide bridge is clearly required for FemAB to be active, i.e. for FemA at least one and for FemB at least three Gly need to be present (Fig. 34).

The enzymatic activity of an individual FemXAB peptidyltransferase does not require the presence of a second species, and the formation of a stable Fem-multienzyme complex is not necessary to achieve a turnover rate *in vitro* that is close to the *in vivo* situation. However, *in vivo*, production of byproducts as observed during *in vitro* synthesis upon increased incubation time and elevated enzyme concentration must be prevented, and it seems reasonable to assume that the interpeptide bridge formation is a highly co-ordinated process, tightly interlinked with the other membrane-associated steps of cell wall biosynthesis (Fig. 17). Possibly, the Fem proteins are transiently associated with each other as well as with other cell wall biosynthesis enzymes. Synthesis of a complete lipid II-Gly_5

may be further favoured in the *in vivo* situation with ongoing translocation of the completely modified cell wall precursor to the outside of the cell. One possible candidate catalysing the flipping of lipid II-Gly_5 to the outside is the above mentioned hypothetical flippase FtsW, a protein associated with the division apparatus complex. Further components with as yet unidentified functions may contribute to co-ordinating the intracellular and extracellular processes to ensure the proper formation of the vital bacterial cell wall. Especially ORF1055, encoded by a gene co-transcribed with *fmhB* and predicted to have 12 putative transmembrane domains as well as two large extracellular domains, would be an interesting candidate for such a role. ORF1055 is most likely essential as well, however, its function remains to be attributed (322). Proteins sharing up to 59 % similarity with ORF1055 belong to the widely distributed RND (resistance-nodulation-division) family of proteins, a ubiquitous group of transmembrane efflux transporters, which have been reported to function in eukaryotes as enzymes or receptors, too (382).

4 OUTLOOK I

The FemABX peptidyltransferases are interesting drug targets for several reasons; i) they are essential, ii) their product is unique and specific for *S. aureus*, iii) blocking of their activity would very unlikely affect the host, since no comparable reaction exists in eukaryotes. Furthermore, their inhibition confers sensitivity for a variety of antibiotics and restores susceptibility towards methicillin (218, 224). Using the here developed *in vitro* assay, the peptidoglycan synthesis in *S. aureus* will be further elucidated. On one hand, cross-linking experiments should reveal whether the Fem factors come close for adding Gly to lipid II in this system. By adding purified $tRNA^{Ser}$-synthetase of *S. aureus* and the immunity factors Lif and Epr of *S. simulans* biovar *staphylolyticus* and *S. capitis*, respectively, we will try to determine whether incorporation of Ser at third and fifth position requires interaction with FemAB factors and, if yes, whether it occurs simultaneously with Gly incorporation or not. Assuming that interaction among the FemAB factors is equivalent to interaction of FemA or FemB with Lif/Epr, it can be thereby extrapolated whether dimerisation occurs and how the length of the interpeptide is determined. For this work, financial support from the “Theodor und Ida Herzog-Egli” as well as the “EMDO” foundation has been accorded. As the development of the described *in vitro* assay system was carried out in very fruitful collaboration with the group of H.G. Sahl, University of Bonn, Germany and with A. Tossi, University of Trieste, Italy, it will be continued.

A long-term project would be the development of an *in vitro* assay using vesicles instead of micelles. This would allow the study of cytoplasmic, membranous and extracellular processes of peptidoglycan synthesis. Of greatest interest, not only for *S. aureus*, would be the identification of lipid II flippases and coordinator proteins, which most likely need a double lipid layer for being active. Once such a “natural” system will be established, testing of putative inhibitors might reveal new approaches for combating pathogens with branched peptidoglycan, not only *S. aureus*, but also pneumococci and enterococci.

5 RESULTS II

5.1. Analysis of the *hemB* mutants MS17 and MS62

To study the interrelationship of global regulators and the role of SigB in a stable mutant mimicking the SCV phenotype, a set of isogenic strains with an interrupted *hemB* gene was constructed in a strain Newman background. *hemB*, which encodes the aminolevulinic acid dehydratase, is essential for hemin synthesis (187). Use was made of the *hemB* gene disrupted by a 1.4 kb fragment containing an *ermB* cassette from the strain 8325-4 derivative I10 (397), which has been well characterised (19, 20, 391). Strain MS17 was obtained by transducing the *hemB* mutation with phage 85 (27) into the Newman derivative MB79 (Fig. 35). This strain contains an integrated reporter construct carrying the firefly luciferase gene (*luc*+) fused to three solely SigB dependent alkaline shock protein 23 promoters (P*asp23*) (129). Strain MS62 was created by transducing the *sigB1* (Am) mutation into the Newman derivative III33 (*hemB*::*ermB*) (Fig. 35) (37, 184). PCR and Southern blot analysis of the *hemB* region of MS17 confirmed that only the desired modification of the *hem* operon had occurred. PFGE-patterns of SmaI-digested DNA from the *hemB* mutants MS17 and MS62 compared to the corresponding parent strain were identical.

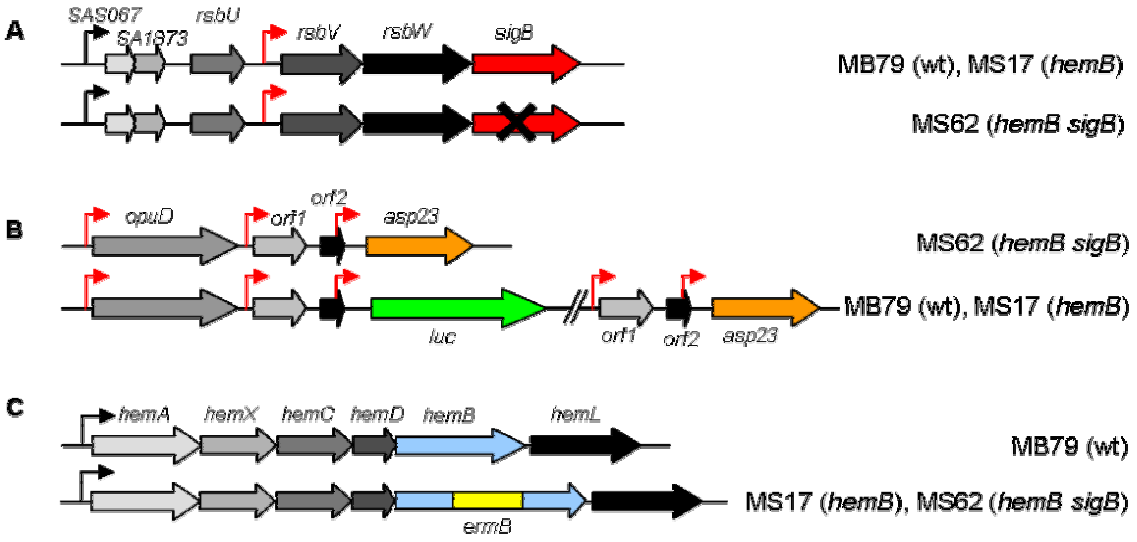


Fig. 35. Relevant loci of strains MB79 (wt), MS17 (*hemB*) and MS62 (*hemB sigB*). **A** *sigB* operon, where a 2 bp deletion in *sigB* lead to a premature stop and a non-functional SigB protein (37). **B** *asp23* operon, into which a plasmid, carrying a fusion of the two proximal *asp23* promoters to the reporter gene *luc*, had been integrated by a single cross over mechanism (129). **C** *hemB*, contained within the *hemAXCDBL* operon, was interrupted by a *ermB* resistance cassette (397). SigA-dependent promoters, black; SigB-dependent promoters, red.

While MB79 reached an OD_{600} of 10 in LB, both the mutants MS17 and MS62, attained a maximal OD_{600} of 0.8 (Fig. 36). The *hemB* mutants entered post-exponential phase later compared to MB79, i.e. after five hours instead of three. Supplementation with 1 μg/ml hemin conferred a normal phenotype to the *hemB* mutants, as determined previously (397).

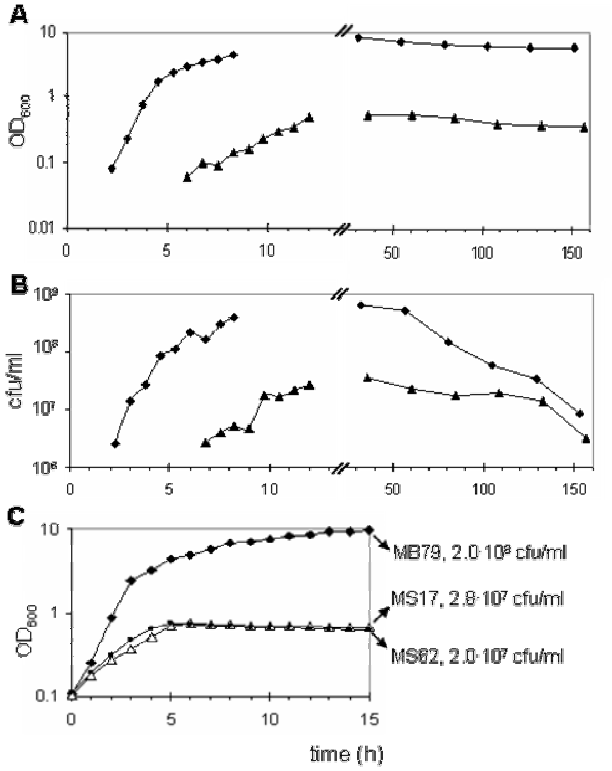


Fig. 36. Growth and viability of MB79 (wt), MS17 (*hemB*) and MS62 (*hemB sigB*). **A** Growth curves and **B** colony forming units (cfu) of cultures, inoculated with overnight cells and an initial OD_{600} of 0.01, were monitored over 7 days. **C** Growth curves from cultures with a ten times higher inoculum (OD_{600} of 0.1) than in **A** and **B**, using washed overnight cells. Cfu/ml were determined after 15 hours. MB79, diamonds; MS17, triangles; MS62, dots. Cfu were determined by spreading appropriate culture dilutions on selective plates.

MS17 and MS62 formed tiny colonies of 0.1 mm on Müller-Hinton (MH) plates, whereas on sheep blood agar cells could apparently scavenge enough hemin to grow up to normal-sized (1 mm), hemolytic colonies (Fig. 37). Viability was not reduced in the *hemB* mutants as judged from colony forming units (cfu) determined over several days (Fig. 36).

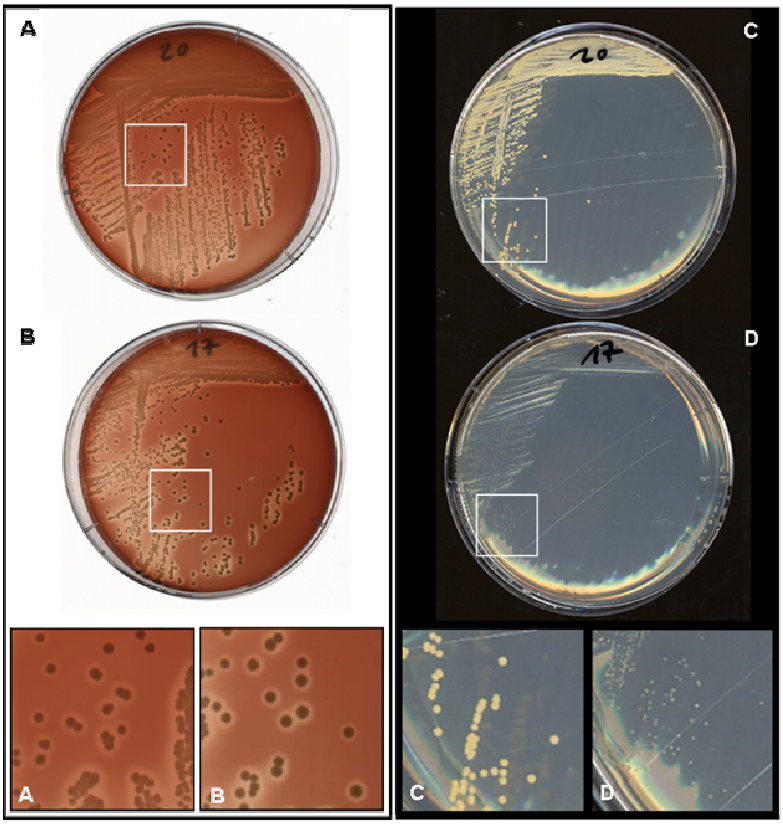


Fig. 37. Colony morphology of strains MB79 (wt) and MS17 (*hemB*). Bacteria were streaked on sheep blood agar (**A**, **B**) or Müller-Hinton agar (**C**, **D**). MB79, **A** and **C**; MS17 **B** and **D**. Magnification of insets is shown below.

Etests on MH medium showed that MS17 and MS62 were more resistant towards gentamicin (4-fold) and amikacin (8-fold) compared to the parent strain. Susceptibility for vancomycin did not differ. Unexpectedly, MS17 and MS62 were more sensitive for oxacillin and teicoplanin by a factor two, respectively four (Table 2). Although higher resistance against cell-wall active antibiotics is often encountered with SCVs, not all the SCVs necessarily display this feature and strain singularities might even lead to a slightly opposite effect (20, 350).

TABLE 2. Antibiotic susceptibility of strains MB79 (wt), MS17 (*hemB*) and MS62 (*hemB sigB*).

	MIC [μg/ml]		
	MB79	MS17	MS62
Amikacin	4	32	32
Gentamicin	1	4	4
Oxacillin	0.38	0.19	0.19
Teicoplanin	3	0.75	0.75
Vancomycin	2	2	2

Growth phase disparity between slow and normally growing cells makes interpretation of temporal profiles difficult. In cultures with an initial OD_{600} of 0.01, the lag phase of *hemB* mutants was more than 4 hours longer compared to that of the parent strain and without a characteristic transition via a post-exponential phase as seen in the parent (Fig. 36). To overcome the growth phase discrepancy, cultures were started with a relatively high inoculum of OD_{600} 0.1, which synchronised the beginning of wt and *hemB* mutant exponential phases (Fig. 36) without influencing their growth phase dependent pattern of gene expression (see *agr* and *sar* profiles below). Still, *hemB* mutants entered the post-exponential growth phase two hours later than their parent. Cells were therefore more frequently sampled in the beginning to obtain representative profiles for both strains and to allow comparison of the temporal gene expression profiles from the respective growth phases. Special care was taken to avoid carry-over of extracellular signal molecules, which might mask growth-dependent processes, by washing cells before inoculation. Growth was monitored over 15 h to include late stationary phase data. To exclude that contamination or reversion had occurred during cultivation, colony forming units (cfu) were routinely determined for all strains on selective and blood agar plates after 15 h; faulty cultures were not used for analyses.

5.2. Transcription of the major regulators *agr*, *sar* and *sigB*

agr expression is initiated in late exponential phase. In the parent strain MB79 RNAII (3.2 kb) preceded RNAIII (0.5 kb) as expected (Fig. 38). In the *hemB* mutant MS17, RNAIII was missing as reported before for *hemB* mutants in strains of 8325-4 and COL backgrounds (205, 391). However, contrary to what Vaudaux et al. observed (391), RNAII was absent as well (Fig. 38). Complementation of the growth medium with hemin restored the *agr* profile in MS17 to the wild type pattern, ruling out an unrelated deletion or mutation affecting *agr* (data not shown).

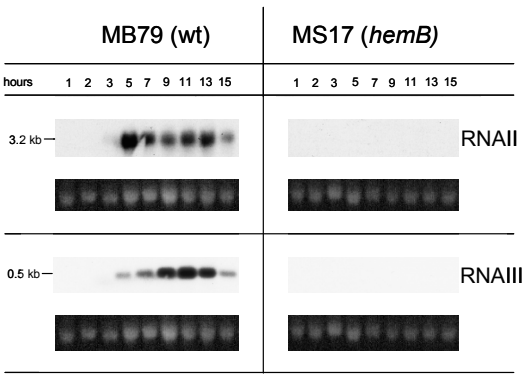


Fig. 38. Northern blot analyses of the two component system *agr* (RNAII and RNAIII) in MB79 and MS17 (*hemB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

The wild type strain MB79 showed characteristic *sar* profiles (21, 39, 239): *sarA* (0.5 kb) and a weak *sarB* (1.2 kb) transcript were mainly present in exponential phase, while the SigB-dependent *sarC* (0.8 kb) transcript appeared in mid-exponential phase reaching a maximum intensity in post-exponential phase (Fig. 39). Interestingly, in the *hemB* mutant MS17 all three *sar* transcripts were maximally expressed in the beginning and were strongly increased compared to MB79. While *sarA* and *sarB* were restricted to the exponential phase, *sarC* was transcribed throughout growth, although slightly decreasing towards stationary phase. The initially enhanced and continued *sarC* transcription directed our interest towards SigB, since *sarC* transcription is exclusively initiated by SigB (61, 126, 129, 170, 214). The *sarC* transcript was confirmed to be SigB-dependent in the *hemB* background as well, since it was abolished in the *hemB sigB* double mutant MS62 (Fig. 39).

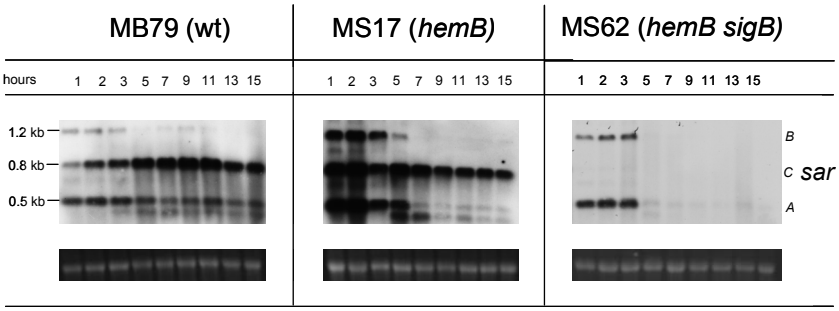


Fig. 39. Northern blot analyses of the SigB-influenced *sar* locus in MB79, MS17 (*hemB*) and MS62 (*hemB sigB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

Transcription of the *sigB* operon is initiated by two SigA-dependent promoters (P1 and P2) producing 3.6 kb and 2.5 kb transcripts containing *SAS067-SA1873-rsbUVWsigB* (P1-*sigB*) and *rsbUVWsigB* (P2-*sigB*) (M. Bischoff, unpublished). In addition, a 1.6 kb stress-inducible, autoregulated, SigB-dependent transcript covering *rsbVWsigB* (P3-*sigB*) is initiated from a third promoter, P3 (126, 129). In strain MB79, both the 3.6 and the 2.5 kb transcripts were expressed weakly and only during early growth, while the 1.6 kb mRNA displayed a more complex pattern: it peaked in late exponential phase, in early post-exponential phase and surprisingly increased again towards stationary phase (Fig. 40). In contrast, in the *hemB* mutant MS17 the 3.6 kb and the 2.5 kb transcripts were almost undetectable. The 1.6 kb transcript, initially stronger than in the parent, disappeared after the exponential phase. As expected, the 1.6 kb SigB-dependent transcript was absent in the *hemB sigB* mutant MS62 (data not shown).

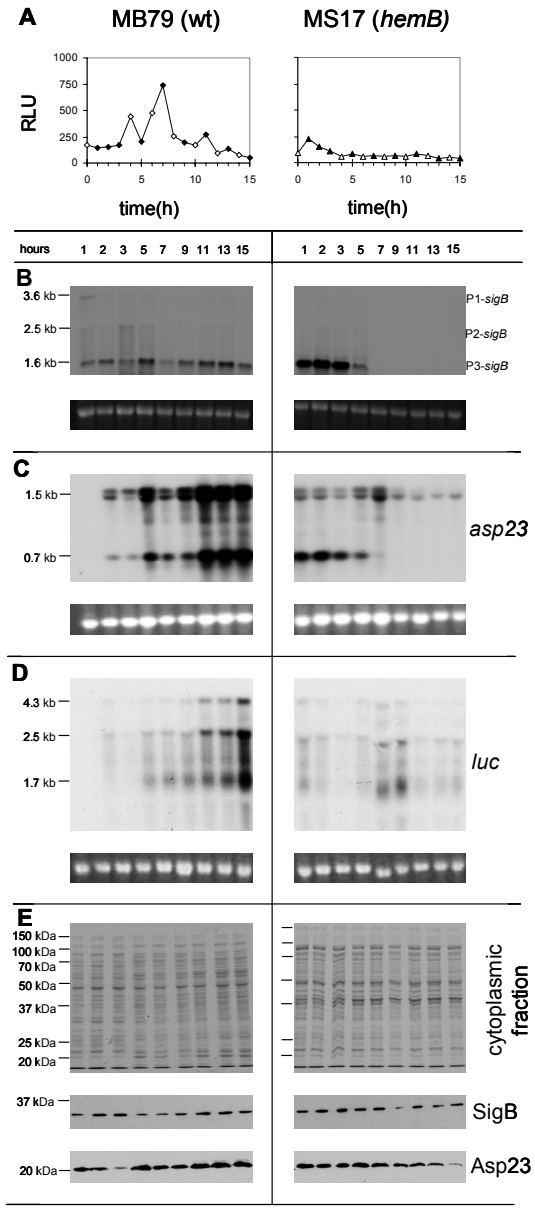


Fig. 40. SigB expression in the Newman background. **A** SigB-activity in MB79 and MS17 (*hemB*) followed by quantifying the amount of luciferase which is transcribed in a SigB-dependent manner from the P*asp23*::*luc*+ reporter construct. Luciferase activity is given by relative light units (RLU) per mg protein of cleared lysate. MB79, diamonds; MS17, triangles. Filled symbols indicate time points of sampling for Northern and Western blot analyses. **B** *sigB,* **C** *asp23* and **D** *luc* Northern blot analyses. The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading. The observed double band at the site of the 1.5 kb *asp23* mRNA might be caused by interference of bulk 16S rRNA. **E** Western blot analyses of SigB (30 kDa) and Asp23 (19 kDa). Cytoplasmic protein fractions were separated by SDS-10% PAGE and stained with Coomassie Blue dye (upper panel). Alternatively, blotted proteins were analysed with antigen-purified anti-SigB antibodies (middle panel) or anti-Asp23 antibodies (lower panel). The molecular sizes of the Precision Plus Protein All Blue Standards (Bio-Rad) marker are indicated on the left.

Despite the difference in transcriptional activity, Western blot analyses of cytoplasmic protein fractions of the wild type MB79 and the *hemB* mutant MS17 revealed that the SigB protein was present at similar, constant levels in both strains throughout growth (Fig. 40). Presence of the SigB protein alone, however, is not necessarily indicative of its activity. As described earlier, the anti-SigB factor RsbW complexes SigB under normal conditions, thereby impeding interaction with the RNA polymerase core-enzyme and consequently transcription of genes preceded by a SigB consensus promoter sequence. Therefore, despite similar levels of SigB protein, there could have been a difference between MB79 and MS17 in SigB activity.

5.3. Expression of the SigB-dependent *asp23* and *luc* genes

A reporter construct consisting of the SigB-dependent promoters of the alkaline shock protein 23 (*asp23*) fused to the firefly luciferase gene (*luc*+) was used to analyse the actual SigB activity (Fig. 35). The parent strain MB79 displayed increased SigB activity in early post-exponential growth phase, with an additional peak at hour 7. In the *hemB* mutant MS17, almost no SigB-activity could be detected by the luciferase assay. Only during the first two hours of growth were relative light unit (RLU) values higher in the *hemB* mutant than in MB79. Thereafter, RLUs dropped quickly below the values measured in the parent (Fig. 40). Western blot analysis of Asp23, which was here under the same control as luciferase, revealed this protein to decrease in the mutant too (Fig. 40), while in MB79 Asp23 seemed to increase from the end of exponential towards stationary phase. Down-regulation of Asp23 in our *hemB* mutant was in accordance with previous findings for a gentamicin-selected SCV (247).

In MB79 and MS17, the resident *asp23* gene is preceded by two SigB-consensus sequence promoters producing 0.7 and 1.5 kb transcripts with identical 3' ends (Fig. 35) (129). Both *asp23* transcripts paralleled the expression profile of the 1.6 kb *sigB* mRNA in the parent strain MB79, peaking at five hours and reaching maximal levels during the last three hours (Fig. 40). In the *hemB* mutant, however, only the 0.7 kb *asp23* mRNA paralleled the MS17 1.6 kb P3-*sigB* transcription profile, with transcription stopping after five hours of growth. Surprisingly, the upper *asp23* transcript was present at low levels throughout the 15 hours. Contrary to what luciferase measurements had suggested, these data indicated SigB activity in the mutant. Both transcripts were clearly SigB-dependent, since there were absolutely no *asp23* transcripts in the *hemB sigB* double mutant MS62 (data not shown). SigB-dependent transcription of luciferase took place throughout growth in the *hemB* mutant (Fig. 40); therefore a yet undefined, *hemB*-linked post-transcriptional process must be responsible for down-regulation of luciferase activity. Surprisingly, the P*asp23*-driven *luc* transcripts displayed a slightly different transcription pattern than the *asp23* transcripts, with peaks at 7 and 9 hours. None of them stopped being transcribed after 5 hours, as did the 0.7 kb *asp23* transcript. Although they were under the control of identical SigB-dependent promoters, for yet unknown reasons *asp23* and *luc* transcription seemed to be regulated by different, additional factors.

The reduction of Asp23 levels over time in MS17 was significantly slower than the corresponding decrease in luciferase activity. Luciferase is known to be rather unstable with a half-life of two hours, making it suitable as a reporter system for detecting fluctuating gene expression (172). In contrast, chloramphenicol acetyl-transferase for example, with a half-life of 50 hours, accumulates and can not reflect changes in transcription (374). Higher Asp23

protein levels compared to luciferase might be due to intrinsic increased protein stability, although still reflecting fluctuations in *asp23* transcription (Fig. 40). This might also explain the relatively high amount of Asp23 in exponential phase in MB79, compared to *asp23* transcription, by a carry over effect from inoculating cells. Availability of ATP within the SCVs as the reason for the slight discrepancy observed between luciferase activity and Asp23 content could be ruled out, as luciferase measurements were performed with cell extracts in excess of ATP.

Interestingly, in the wild type strain MB79, the increased SigB-dependent transcription of *luc* after nine hours (Fig. 40) lead to only minor peaks in luciferase activity (Fig. 40). Whether this inconsistency is due to a similar physiological state of wild type stationary phase cells and SCVs remains to be determined. Preliminary long-term monitoring of luciferase activity further supported the observation that this reporter system does not reflect SigB activity after 10 h (appendix 9.3., Fig. 52), since auto-regulated *sigB* transcription seemed to occur in a fluctuating way during 7 days, without being reflected in luciferase activity.

5.4. Transcription of SigB-regulated genes

Due to the apparent poor performance of the luciferase reporter system in the *hemB* mutant, SigB activity was measured directly by monitoring transcription of SigB-controlled genes such as *clfA* and *fnbA* by Northern blot analyses (38, 272).

The *clfA* gene is preceded by a SigA- and a SigB-dependent promoter, which initiate transcripts of 3.7 kb and 2.9 kb, respectively (100, 272). The 3.7 kb SigA-dependent transcript was seen in all three strains during exponential growth phase, irrespective of the changes triggered by *hemB* or *sigB* inactivation (Fig. 41). The SigB-dependent 2.9 kb *clfA* mRNA roughly followed the 1.6 kb P3-*sigB* transcription profile in MB79, with peaks at 3 and 13-15 h. Main expression of *clfA* in late growth phase was in accordance with previously made observations (38, 100, 409). In the *hemB* mutant MS17 however, the 2.9 kb *clfA* transcript displayed a quite different transcriptional pattern than the 1.6 kb P3-*sigB* mRNA. After a strong initial signal, transcription diminished, and then increased again towards stationary phase, comparable to what was observed in the parent strain. In the *hemB sigB* double mutant MS62 the 2.9 kb *clfA* mRNA was absent, demonstrating its SigB-dependence in strain MS17.

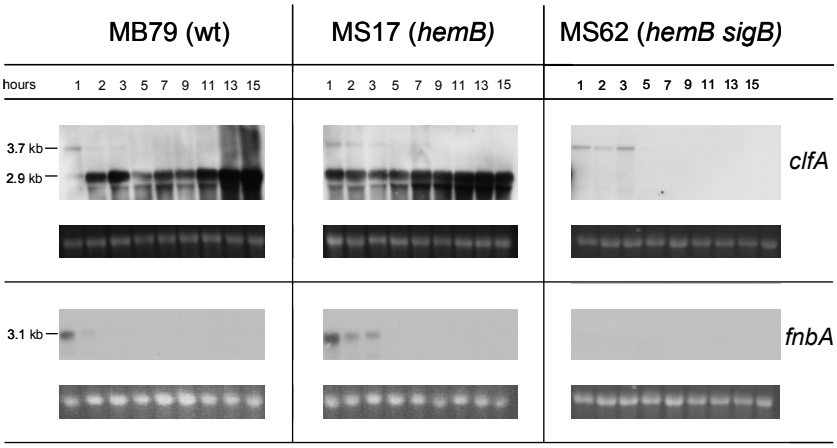


Fig. 41. Northern blot analyses of the SigB-influenced genes *clfA* and *fnbA* in MB79, MS17 (*hemB*) and MS62 (*hemB sigB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

Although not preceded by an apparent SigB consensus promoter sequence, the *fnbA* gene is positively influenced by SigB (38, 100, 272, 415). While the recently reported, SigA-dependent 4.5 kb *fnbA* transcript (100) was not observed in the Newman background (data not shown), a 3.1 kb *fnbA* transcript was detected during the first hours after inoculation in MB79 and MS17, but was completely missing in the *hemB sigB* mutant MS62 (Fig. 41). In the *hemB* mutant MS17, *fnbA* transcription was slightly prolonged compared to the parent strain MB79. Transcription of *fnbA* mainly in early exponential phase was in agreement with previous reports (38, 100, 415).

The presence of SigB-dependent *asp23*, 2.9 kb *clfA, fnbA* and *sarC* transcripts in the *hemB* mutant MS17, and their respective absence in the *hemB sigB* mutant MS62, proved that SigB was required and active at all time points in MS17. Nevertheless, in this strain, differences in the extent of stimulation of the directly SigB-regulated genes were observed, which might be attributed to the involvement of additional regulators other than SigB.

5.5. mRNA stability of *sarC* and 2.9 kb *clfA*

Another explanation might be that the mRNA half-lives of these SigB-dependent transcripts may vary; in which case the 2.9 kb *clfA* and *sarC* transcripts would be the most stable. However, there was neither a significant difference in mRNA stability between parent and mutant, nor between *sarC* and 2.9 kb *clfA* (Fig. 42). The approximate half-life of transcripts was 3 minutes, which was similar to values determined for the *asp23* transcripts (M. Bischoff, unpublished results) and too short to persist over hours. As a comparison, half-life for *norA* mRNA of *S. aureus* is 7 minutes (117). The half-life of the majority of *B. subtilis*

mRNAs has been determined to be below 7 minutes, that of 90 % of *S. aureus* mRNA is below 5 minutes (91, 152).

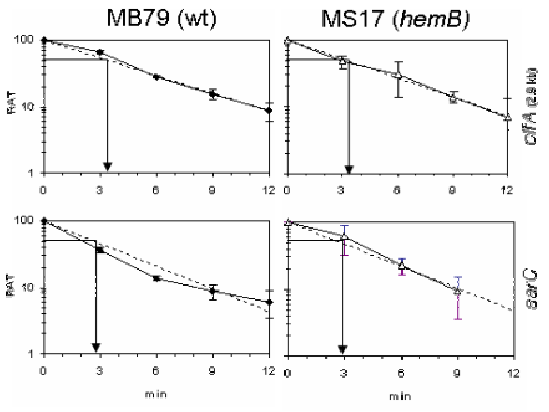


Fig. 42. Stability of 2.9 kb *clfA* and *sarC* mRNAs in MB79 (wt) and MS17 (*hemB*). The relative amount of transcript (RAT) is expressed as a percent of the quantity at the time of rifampin addition. Regression lines are based on mean values from at least two independent experiments.

5.6. Transcription of global regulators

S. aureus is known to produce a vast array of regulatory elements that allow the pathogen to fine tune its transcriptional profile in response to environmental stimuli. To gain a more comprehensive view of regulation in MB79 and its *hemB* mutant MS17, regulators which are important for virulence, have been reported to influence the genes altered in SCVs or are involved in cellular processes likely to play a role in the SCV phenotype were monitored. Surprisingly, the hereafter described regulatory elements were only transcribed in exponential phase in the *hemB* mutant MS17, contrasting with individual profiles generally spanning the entire monitored time in the parent strain MB79.

5.6.1. Two component systems

Previous findings about *arl*, *sae* and *srr* expression were basically confirmed in MB79 (Fig. 43):

The 2.7 kb *arl* transcript was seen throughout growth and increased slightly in the late stationary phase, paralleled by a weaker band of 1.5 kb probably encoding *arlS* alone (116).

Of the three overlapping *sae* transcripts, containing *saePQRS* (3.1 kb), *saeQRS* (2.4 kb), or *saeRS* (2 kb), *saeQRS* was the most prominent and peaked in the late post-exponential phase. A fourth transcript starts at the same promoter as the 3.1 kb transcript but ends at a stem loop sequence after 0.7 kb; it only contains *saeP* and basically paralleled the profile of the other three mRNAs (data not shown) (135, 284, 363). Variations in transcription profiles exist for different genetic backgrounds, for example the 2 kb transcript is hardly

detectable in Newman (363). However, overall *sae* transcript levels are highest towards stationary phase of growth, as seen in MB79 as well.

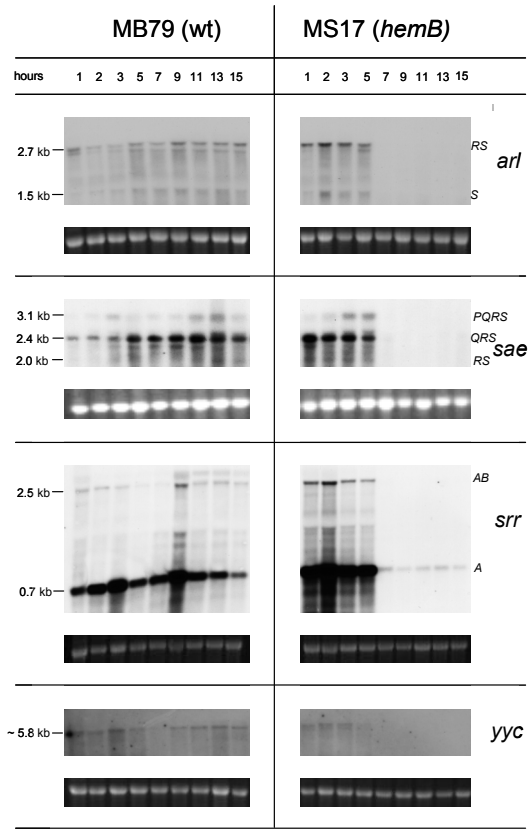


Fig. 43. Northern blot analyses of the two component systems *arl*, *sae*, *srr* and *yyc* in MB79 and MS17 (*hemB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

The weaker 2.5 kb *srrSR* and the generally stronger 0.7 kb *srrA* mRNA, reported to be produced until post-exponential phase (418), showed peaks here at 3 and 9 h and were expressed throughout the 15 h monitored.

The profile of *yyc* was similar to that of *arl*. A transcript of approximately 5.8 kb was observed, suggesting that *yycFG* was cotranscribed with at least two of the yet uncharacterised downstream genes. In *B. subtilis*, promoter fusion experiments monitoring transcription of *yycFG* during 6 hours (102), indicate transcription in early growth; data for *S. aureus* were not published until now.

arl, *sae* and *srr* transcription were up-regulated in the *hemB* mutant MS17 during exponential growth and disappeared thereafter except for a very faint band of *srrA* was detected throughout growth. *yyc* was expressed at comparable levels to those in MB79 (Fig. 43).

5.6.2. SarA homologues

The *rot* transcript has been reported to be present throughout growth (258, 336). In MB79, *rot* mRNA of approximately 0.6 kb was maximally expressed in post-exponential phase and declined in stationary phase, similar to the 0.4 kb *sarR* mRNA (Fig. 44), whose transcription profile had been analysed up to early stationary phase (238).

In an 8325-4 background a 0.95 kb SigA-dependent transcript of *sarS* had been found to be present mainly in early exponential growth. Upon overproduction of SigB, a 1.55 kb mRNA initiated from a SigB-promoter consensus sequence is produced, paralleled

by a 0.8 kb mRNA possibly a processing product of the larger transcript (372). In the Newman background *sarS* transcription is however not affected by SigB, as determined by microarray analyses (38). A 0.8 kb *sarS* transcript in MB79 was found in low amounts mainly in early exponential and towards stationary phase (Fig. 44).

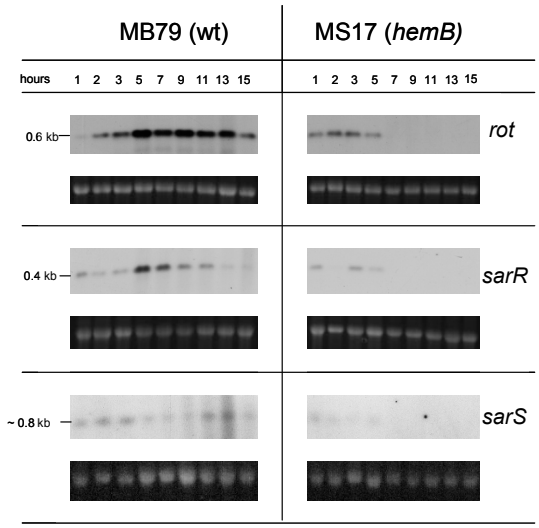


Fig. 44. Northern blot analyses of the SarA homologues *rot*, *sarR* and *sarS* in MB79 (wt) and MS17 (*hemB*). Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading. The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

In the *hemB* mutant MS17, *rot*, *sarR* and *sarS* transcription were reduced compared to MB79 and absent after 5 hours.

5.6.3. The regulators SvrA and TcaR

RT-PCR has shown that *svrA* is expressed in post-exponential phase. However, there is no information available on the size of the transcript(s) or their temporal pattern (124). A very faint transcript of approximately 2.5 kb was detected in both strains only during early growth, suggesting that *svrA* (1.35 kb) is co-transcribed with the surrounding genes of yet unknown function (Fig. 45). Paralleling the larger transcript, a shorter, approximately 1.5 kb transcript might be present. In MS17, the transcripts were almost undetectable. At the height of 23S rRNA, a stronger band was observed, which however is most probably due to unspecific binding.

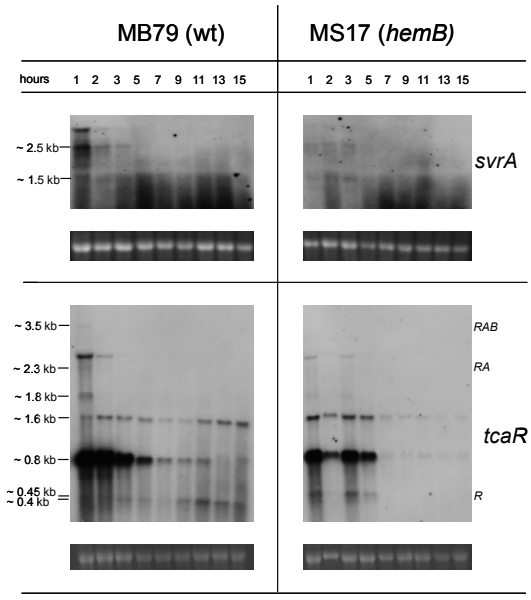


Fig. 45. Northern blot analyses of the virulence regulator *svrA* and the *tcaR* transcripts in MB79 (wt) and MS17 (*hemB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

In MB79, *tcaRAB* (3.5 kb), *tcaRA* (2.3 kb) and an un-assigned band of 1.8 kb were only present in early growth phase. *tcaR* (0.45 kb) was present throughout growth, with an apparent decrease around hour seven (Fig. 45). An additional band of 0.8 kb which might comprise *tcaR* and a part of *tcaA*, was extremely strong early in growth but then diminished towards stationary phase. The *tcaR* probe used does not hybridize to the *tcaA* transcript (1.6 kb), which has been shown to be almost undetectable under normal growth conditions, although it is strongly induced by teicoplanin (236). Therefore, the two bands observed at both the 23S and 16S rRNA height are most probably due to unspecific binding of the probe to the respective nucleotides. In the *hemB* mutant MS17, transcription was generally reduced in exponential phase and almost undetectable thereafter.

5.7. Transcription of the virulence determinants *spa*, *hla* and *isaA*

spa is generally transcribed slightly earlier in the exponential phase, followed by *hla* (92, 372, 390). Most of the above described regulators influence directly or indirectly either *spa* or *hla* expression. In MB79, *spa* was maximally transcribed in post-exponential phase; *hla* transcription started shortly after *spa*, but reached its maximum level earlier and was thereafter rather constant (Fig. 46).

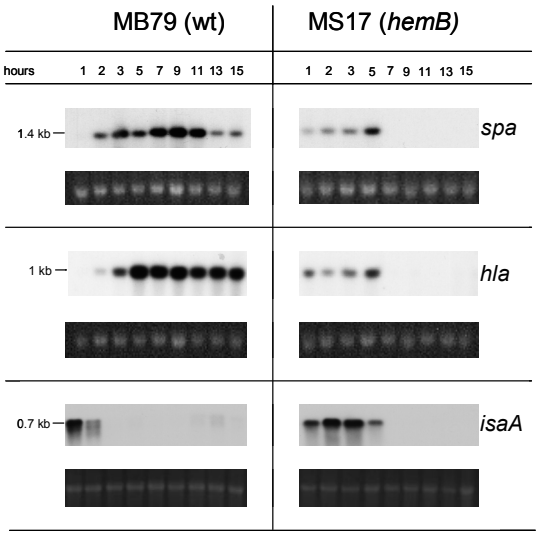


Fig. 46. Northern blot analyses of the virulence determinants *spa*, *hla* and *isaA* in MB79 (wt) and MS17 (*hemB*). The sizes of relevant bands are given on the left. Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

isaA expression has been reported to occur during early growth (38), which was confirmed for MB79. Transcription of *isaA* was prolonged and increased in the *hemB* mutant MS17 compared to MB79, in a similar fashion to *fnbA* (Fig. 41).

6 DISCUSSION II

6.1. Rationale for the applied methods

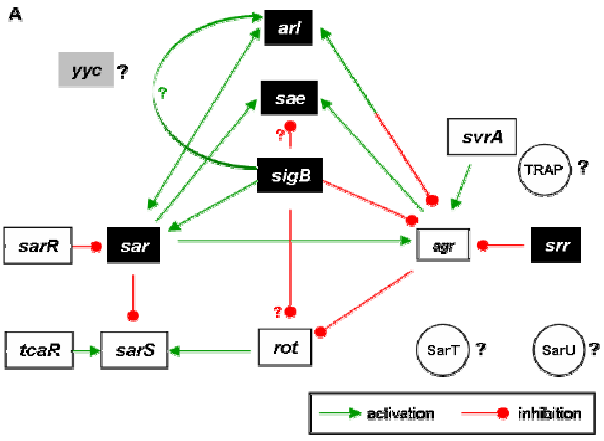
S. aureus SCVs recovered from clinical specimens and SCVs selected by gentamicin exposure are genetically undefined and may carry mutations in more than one site, especially since these strains show multiple phenotypic changes compared to the parent strains. In addition, clinical SCVs exhibit an unstable phenotype and easily revert to wild-type phenotype during cultivation. These inconveniences can be circumvented by disruption and inactivation of hemin or menadione synthesising enzymes. Several studies involving *hemB* mutants, including the comparison of 8325-4 and COL *hemB* mutants with clinical SCVs, had previously shown that these mutants mimic the SCV phenotype during prolonged *in vitro* and *in vivo* growth (20, 54, 184, 333, 397). Although no information about the molecular characterisation of clinically emerged *S. aureus* hemin-auxotrophic SCVs is available, *in vitro* gentamicin-selected SCVs did carry mutations in the *hemAXCDBL* and *hemEHY* operons (343). Moreover, a clinical isolate of Gram-negative SCVs had been found to carry a deletion of *hemB*, further indicating that defects in hemin synthesis are relevant for *in vivo* SCV formation (320, 321).

The laboratory strain Newman was chosen because this frequently used strain has an intact *sigB* operon and had been reported to produce large amounts of FnBPs, proteins up-regulated in SCVs (93, 363). Only recently point mutations in both *fnbA* and *fnbB* were found, leading to a truncation of the FnBPs and making this strain unsuitable for pathogen-host interaction studies. However, transcriptional regulation of *fnbAB* is very unlikely affected (149).

Differences in the genetic background as well as minor changes in growth conditions can have major impacts on the profile of a regulator. Temporal transcription patterns stemming from few time points easily miss fluctuations occurring in between. Published results are therefore not always connectable. Hence, this study provides a useful overview of wild type transcription profiles of the most important regulators. New information for several regulators was presented (*sarR*, *svrA* and *yyc*). In addition, the experiments confirmed previous findings concerning the transcriptional patterns of *agr*, *arl*, *rot, sae*, *sarS*, *srr*, *tca* and *sigB,* while completing their data regarding stationary phase expression.

6.2. Altered gene expression in the *hemB* mutant

The temporal pattern of the analysed global regulators in the *hemB* mutant did not reflect any particular growth phase of the parent strain, nor was it attributable to the absence or predominance of one single regulatory element. Deviations from the wild-type pattern were found for all global regulators and could be classified in three groups: i) no transcription, as observed for *agr*, ii) transcripts that were only present until cells entered post-exponential phase; during this time they were either up-regulated (*arl*, *sae*, *sarA, sarB, sigB* and *srr*), down-regulated (*rot*, *sar*R, *sar*S, *svrA* and *tca*) or similarly expressed as in the parent strain (*yyc*), and iii) transcription throughout the observed time course, as seen for *sar*C. In the general model of regulator interplay (Fig. 2) these relative overall transcript levels were indicated in Fig. 47, serving as an orientation for the following discussion.



B

target	+ influenced by							- influenced by
asp23		sigB						?
clfA		sigB						?
fnbA	sae	sar	sigB					agr
hla	sae	sar	svrA	agr	arl	sigB	rot	sarS
isaA	sar	sigB	yyc ?		yyc ?			
spa	tcaR	rot	sarS	sar	arl	srr	svrA	agr

Fig. 47. Interplay of global regulators and regulation of virulence determinants in strain Newman and its *hemB* mutant. Higher (black boxes), similar (grey boxes) or lower overall transcription levels (white boxes) during exponential phase in the *hemB* mutant compared to the parent strain are indicated. **A** Regulatory network. **B** Target genes and factors influencing their expression.

6.2.1. *agr*

The complete absence of the major regulator *agr* in the Newman *hemB* mutant is supposed to have an influence on the expression of several genes. It could explain for instance the lack of *sae* transcription after the exponential phase in the *hemB* mutant since its transcription depends in post-exponential phase on *agr* (135, 284). In contrast, the increase of the adhesion factors FnbA and ClfA was not caused by the absence of RNAIII,

which in the case of *fnbA* seemed feasible, because *agr* is a known inhibitor of *fnbA*. However, up-regulation of *fnbA* (and *clfA*) transcription is reported to occur in a *hemB* mutant of an RNAIII-defective 8325-4 strain as well (391). In both MS17 and MB79 *agr* was not (yet) expressed when *fnbA* transcription began to decline. These findings support the idea that some additional repressor of *fnbA* must exist (411). *arlRS* was up-regulated in the *hemB* mutant in exponential phase compared to the parent, probably by SarA, because the other known stimulator, *agr*, was absent. Since it has been shown that *arlRS* depends both on *sar* and *agr* for *spa* down-regulation (116), *arl* might only have contributed to down-regulation of *agr* (thereby forming a negative feedback loop) and *hla* in the *hemB* mutant.

Preliminary long term monitoring experiments over 7 days revealed both RNAII and III being transcribed in the *hemB* mutant after 30 hours (appendix 9.3., Fig. 52). In addition, both *agr* transcripts could be detected during the exponential phase in unrelated, menadione-auxotrophic clinical SCV isolates (appendix 9.4., Fig. 53), showing that, despite the low cell density, *agr* transcription can be induced in SCVs. Together, these findings indicate that in *hemB* mutants and SCVs, depending on the strain's genetic background, differences in *agr* regulation exist. Therefore, absence of *agr* upon entry into post-exponential phase might not be a common characteristic of SCVs, although in the Newman background analysed here it certainly influenced other genes.

SvrA has been proposed by Garvis et al. to play a role in transducing the extracellular RAP signal to the cytosolic TRAP protein (124), which upon phosphorylation becomes activated and up-regulates the *agr* locus by a yet unknown mechanism (145, 208). This model would favour an early expression of *svrA*, as observed here. Interestingly, the stop of the 2.5 kb *svrA* transcription nicely coincided with the start of *agr* expression in the wild type. However, due to low transcription levels of the hypothetical 1.5 kb mRNA (Fig. 45) containing *svrA* alone, its transcription pattern was difficult to determine, for which we can not exclude the presence in post-exponential phase, as previously found (124). No information concerning the function of the neighbouring genes was available to date. While the existence of an SvrA-RAP-TRAP network still awaits prove, the reduction of ATP encountered in the *hemB* mutant could indeed hamper such a phosphorylation-dependent signal transduction pathway, not only in the case of the *agr* locus.

6.2.2. *sar*

In the above mentioned study, *sar* transcription in an 8325-4 *hemB* mutant had been shown to be reduced (391). While the overall *sar* transcription was found to be increased in exponential phase in the *hemB* mutant, thereafter the amount of transcripts decreased to similar or lower levels compared to the parent. However, by using Northern blot techniques

and by monitoring the entire growth cycle a more detailed image was obtained. In the *hemB* mutant, *sarC* was the only regulator encoding transcript present throughout growth and, in consequence, its exclusive and required activator SigB was present and active as well. The strongly reduced *sarR* transcript levels in the *hemB* mutant MS17 could lead to decreased SarR activity, potentially contributing to increased SarA levels as shown in a *sarR* mutant (238).

Since findings of reduced *sar*, but increased *fnbA* and *clfA* transcription in an 8325-4 *hemB* mutant stem from just one time point of late-log phase (391), comparison with our results is difficult. Evaluating mRNA levels during late exponential phase in MB79 (2 and 3 h) and MS17 (3 and 5 h), increased *fnbA* transcription were found in the Newman *hemB* background as well, however, overall *sar* mRNA levels were increased while *clfA* seemed to be lower in late-log phase (Figs. 39 and 41). Apart from sampling differences, the diverse genetic background might have had an influence on relative expression levels as well, as seen for *agr*.

In the wild type strain, at all time points either *sar* or *agr*, or both, were supposedly present, thereby assuring *sae* transcription throughout growth (Fig. 43). Since the only known activator expressed in the *hemB* mutant was *sar*, it might have been responsible for *sae* activation, similarly as for *arl*. Alternatively, an unknown activator could have played a role.

A sequence upstream of the SarA coding region has been reported to be essential for *fnbA* transcription and is contained in *sarB* and *sarC* transcripts, but not in *sarA* (411). Since in the *hemB* mutant MS62 *sarB*, but neither *sarC* nor *fnbA* were transcribed, it looked as if only *sarC* could provide the required elements for *fnbA* expression. Still, this could not have been the case in the wild type, where transcription of *fnbA* started before *sarC* was present. Possibly *fnbA* transcription requires SigB and either *sarB* or *sarC*. However, *fnbA* regulation might be much more complicated, since *sae*, *sar* and *sigB* have been reported to be equally required for *fnbA* expression (38, 272, 363, 411).

6.2.3. Virulence determinants

isaA seems to be directly regulated by the response regulator YycF, although it has not been reported whether positively or negatively. While *yycFG* mRNA levels during exponential phase were similar, transcription of the target *isaA* was much more pronounced during the first five hours in MS17 compared to MB79 (Fig. 43 and 46). SarA and SigB have been shown to have a positive influence on *isaA* (38, 86, 425, 426). Together, these regulators might be responsible for the up-regulation of *isaA*.

The only known direct activator of *spa* is SarS, which in turn is positively regulated by Rot, SarT and TcaR, and negatively influenced by SarA (253, 336, 346, 372). Apparently, although relatively weakly transcribed, SarS dominated over ArlR, SarA, SrrA and RNAIII in the wild type, all of which have been reported to negatively influence *spa* transcription (21, 23, 115, 116, 175, 309, 418). In the *hemB* mutant MS17, decreased *spa* levels suggested that SarS was overridden by SarA or SrrA, since ArlR has been shown to require both *sar* and *agr* for its action on *spa* (Fig. 46) (116). In addition, the SarS-activator TcaR and Rot levels were possibly reduced. However, as seen for Asp23, reduced transcription is not necessarily correlated with similarly decreased protein levels, let alone activity.

Regulation of *hla* is more complicated since *sae*, *sar* and *agr* are equally important for its transcription *in vitro* (138). Both ArlR and SigB negatively influence *hla* transcription (38, 116, 129, 426). As discussed for *spa*, activating factors must have prevailed in the wild type MB79; *agr*-independent initiation of *hla* transcription by *sae* (or *sar*) was seen around hour 2 in the wild type, when *agr* had not yet been induced. On the contrary, in the *hemB* mutant MS17 the inhibitors were stronger, although they could not impede weak, *agr*-independent transcription during exponential growth.

6.2.4. *sigB*

Long term follow-up of SigB-dependent transcription revealed that SigB was active with fluctuations until stationary phase in the parent strain, possibly in response to general changes in growth conditions. Surprisingly, SigB-driven expression of known SigB-dependent genes varied significantly in the Newman *hemB* mutant. While *sarC*, the 2.9 kb *clfA* and the 1.5 kb *asp23* transcripts were detectable throughout growth, this was not the case for the 1.6 kb P3-*sigB* and the 0.7 kb *asp23* transcripts. Based on these observations, the presence of factors modulating SigB activity in the recognition of its promoter consensus sequences under certain circumstances is postulated. Such SigB-modulators may also explain diverging results concerning SigB activity in different genetic backgrounds and varying experimental conditions.

Because of the lack of the major SigB-activator RsbU in strains with an 8325 background, SigB activity in these bacteria is generally strongly reduced (39, 129). However, apparently another, yet unidentified activator protein of SigB exists, since some reports about transcription of the *sar* locus revealed that, despite the absence of functional RsbU, SigB-dependent transcription of *sarC* can take place (21, 41), but not always does (39, 157). The factors responsible for induction of this bypass are not yet identified, neither are the mechanisms. Since up-regulation of the SigB-dependent *fnbA* and *clfA* transcripts was observed in an *rsbU* background, one could speculate that in the *hemB* mutant this

alternative SigB activator is induced (391). The signal responsible for enhancing SigB activity in SCVs remains to be determined; a possibility would be energy stress. Glucose starvation has been found to induce SigB activity (M. Rüegg & M. Bischoff, unpublished results). Still, since glucose seems to be depleted later compared to parent strains, as seen in 8325 and COL *hemB* mutants, another, earlier signal must exist for SigB activation (under the assumption that those observations apply to strain Newman as well) (20).

In *B. subtilis*, energy stress has been shown to influence SigB activity by two mechanisms. In the first one, a low ATP/ADP ratio affects RsbW kinase activity in that binding to RsbV is not followed by phosphorylation, leading to higher amounts of RsbW-RsbV aggregates and consequently free SigB. The second mechanism involves an alternative phosphatase, RsbP, which seems to be missing in the *S. aureus* genome. In *B. subtilis*, RsbP becomes activated upon energy stress, dephosphorylates RsbV and tilts the amount of RsbW-SigB towards RsbW-RsbV, increasing levels of uncomplexed SigB (79).

Although *sigB* was not an essential locus for the *in vitro* SCV phenotype, as its inactivation did not affect colony morphology, growth, or antibiotic resistance in MS62, it is nevertheless strictly required for the maximal expression of at least two important virulence factors; FnbA and ClfA. Expression of FnbA, the more effective adhesion and invasion factor of the two redundant FnBPs A and B (149), together with ClfA, which recently has been shown to protect bacteria from phagocytosis by macrophages (292), might significantly contribute to persistence in the host.

As already mentioned, one can not deduce from transcript levels the amount of active protein. However, keeping in mind that SigB was present and active, the observed transcript levels in exponential phase fit relatively well to the model of regulator interplay and regulation of virulence determinants (Fig. 47). For instance, positively SigB-influenced genes were found to be up-regulated or at least expressed at similar levels (*sar*, *asp23*, *clfA*, *fnbA*, *isaA*), whereas negatively influenced genes were down-regulated (*agr*, *hla*). The reduced *spa* mRNA levels correlate with increased inhibitor transcripts (*sar*, *arl* and *srr*). Still, even after assessing the presence and activity of each of these regulators, due to complex mutual interactions, the net result of activation or inhibition can hardly be predicted and needs to be assessed for each target. Factors responsible for the down-regulation of *rot*, *sarR*, *svrA* and *tcaR* remain to be identified.

6.3. General deviation of transcriptional activity in the *hemB* mutant

Although most of the analysed genes' transcription is concentrated in exponential phase in the *hemB* mutant, some of their products were presumably present until stationary phase, as was found for SigB. Results for *fnbA* and *isaA* transcription combined with data reported for FnbA and IsaA strongly suggest that these proteins persist until the stationary stage as well (205, 391). Speculating that the energy-restricted *hemB* mutant does not express needless genes, the increased transcription of *arl*, *sae, sar* and *srr* suggests that these regulators are involved in the control of its transcriptome. A role for *srr* in *hemB* mutants has already been proposed due to the fact that several metabolic genes influenced by *srr* were changed in a COL *hemB* mutant (205). The finding of Kohler et al. that the aconitase *citB*, a TCA-cycle enzyme controlled by *srr*, is down-regulated in a *hemB* COL mutant (205) was confirmed by us. In addition, *srr* could have contributed to *agr* and *spa* down-regulation.

Interestingly, both *spa* and *hla* transcription started earlier in the mutant (Fig. 46). This was also observed for *rot*, *asp23* and 2.9 kb *clfA* transcription (Figs. 40, 41 and 44), arguing against the idea that SCVs are cells stalled in early growth phase, as suggested by the absence of *agr*, exoprotein production and prolonged transcription of genes typically expressed in early growth phase, like *fnbA* (Fig. 41). Whether the almost uniform stop of transcriptional activity upon exit of the exponential phase is a coincidence or correlates to a yet uncharacterised transition process remains open. A link to reduced ATP levels seems difficult to make, since the reduced ATP levels are rather constant in a COL *hemB* mutant (205). Various metabolic enzymes have been reported to be transcribed after the exponential phase, suggesting transcription to focus on selected and required genes (205).

Both the premature transcription and the drastic reduction of transcription observed in the *hemB* mutant, for SigB dependent and independent loci, indicate that global alterations of gene expression do happen. Thus, the extraordinary cellular state caused by the interrupted electron transport in *hemB* mutants is linked to an altered activity of global regulators and expression of virulence factors.

7 OUTLOOK II

A subject of future work would be to determine the presence and activity of the regulators whose transcription was found to be up-regulated during the exponential phase in the *hemB* mutant (*arl*, *sae*, *sar* and *srr*). Results found for Newman and COL backgrounds would have to be confirmed in other types of SCVs; menadione- and thymidine-auxotrophs but also in clinical isolates. Most likely none of the above mentioned regulators will prove to be crucial for the SCV phenotype, as seen for SigB (and *agr*). However, SigB still could influence virulence of these variants, especially by increasing factors needed for adhesion and uptake into host cells, important steps for SCVs to establish themselves and persist. This could be tested by comparing *hemB* with *hemB sigB* mutants in a simple animal model like *C. elegans*. However, a strain other than Newman, expressing functional FnBPs, should be used to study host-pathogen interactions.

The identification of factors modulating SigB activity would be of general interest. Due to the constitutive energy stress, *hemB* mutants might be a useful tool to identify missing factors involved in regulation of SigB activity. Since these additional factors could be enriched in the *hemB* mutant, pull-down experiments of SigB incubated with cell extracts from these bacteria might be a promising approach to find proteins involved in SigB regulation. RsbU activity might be changed as well; identification of interaction partners could bring light into the mode of SigB activation in response to energy stress; a pathway still missing in *S. aureus*.

8 MATERIALS AND METHODS

8.1. Oligonucleotide primers

TABLE 3. Oligonucleotide primers used in this work.

Name	Sequence (5'-3')	Remarks	Reference
Dig-*tcaR*F	CGTTAACGCATTAACTGCAA	*tcaR* DIG-labelled probe, fwd	(236)
Dig-*tcaR*R	GCGATGATTGATGACTTCTA	*tcaR* DIG-labelled probe, rev	(236)
IK14	ACGCGAAGGTGGCCTAG	*sigB* DIG-labelled probe, fwd	(213)
IK15	ATGGTCATCTTGTTGCCCC	*sigB* DIG-labelled probe, rev	(213)
MS14	CGAAGACGATCCAAAAC	RNAII DIG-labelled probe, fwd	This work
MS15	TTATCTAAATGGGCAATGAGT	RNAII DIG-labelled probe, rev	This work
MS16	GGGATGGGACAAGATAAAGAAGC	*fnbA* DIG-labelled probe, fwd	This work
MS17	ACGACACGTTGACCAGCATG	*fnbA* DIG-labelled probe, rev	This work
MS20	AGAAAATGGCATGCACAAAAA	*hla* DIG-labelled probe, fwd	This work
MS21	TGTAGCGAAGTCTGGTGAAAA	*hla* DIG-labelled probe, rev	This work
MS22	GGCAATCAAATCATTCCGGATACTG	*luc*+ DIG-labelled probe, fwd	This work
MS23	ATCCAGATCCACAACCTTCGCTTC	*luc*+ DIG-labelled probe, rev	This work
MS24	CTAGCTAGCGCAAAAGATATGGATAC	*glyS*+, *Nhe*I site, no ATG, for cloning into pET-24b	This work (349)
MS25	CATCTCGAGGAATTTTGTTTTTTCAGTTAAG	*glyS*-, *Xho*I site, no TAA, for cloning into pET-24b	This work (349)
MS26	CGGAGTGCTTAATGCGTTAG	*SArRNA10* DIG-labelled probe, fwd	This work
MS28	AGATCTCGATCCCGCGA	pET-24a(+) fwd, position 342 at *Bgl*II site, for sequencing	This work
MS29	GCTCAGCGGTGGCAG	pET-24a(+) rev, position 80 at *BpU*1102I site, for sequencing	This work
MS30	CAATGGGCGAAAGCCTG	*SArRNA10* DIG-labelled probe, rev	This work
MS31	CATTGCCAATGATGATAGGGAC	*svrA* DIG-labelled probe, fwd	This work
MS33	CGTGGCTTCAGTGCTTGTAG	*clfA* DIG-labelled probe, fwd	This work
MS34	GAGTTGTTGCCGGTGTATTAGC	*clfA* DIG-labelled probe, rev	This work
MS39	CAAGTTTTGGGATTGTTGGGATG	*rot* DIG-labelled probe, fwd	adapted from (336)
MS40	GCTCCATTCATTTGTGCCATAG	*rot* DIG-labelled probe, rev	This work
MS41	CAGCATGGTCTTGCTGC	*sarS* DIG-labelled probe, rev	This work
MS42	CAAGCCTGAAGTCGATATGAC	*sarS* DIG-labelled probe, fwd	This work
MS45	GACCCACTTACTGATCGTG	*saeR* DIG-labelled probe, fwd	This work
MS46	CCTAATCCCCATACAGTTGTG	*saeR* DIG-labelled probe, rev	This work
MS59	CGAAATACTTATCGTAGATGATGAGGATAG	*srrA* DIG-labelled probe, fwd	This work

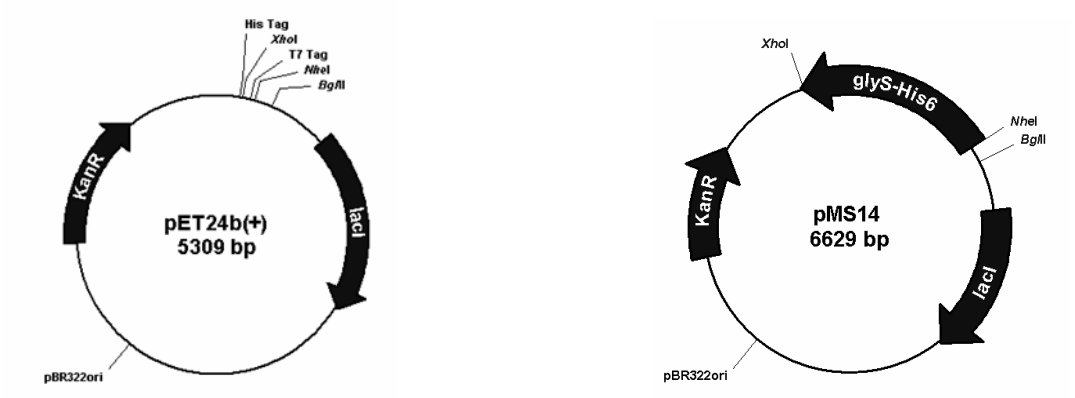
MS60	CAGCAAGTACGCGATGTGC	*srrA* DIG-labelled probe, rev	This work
MS61	GAGTCCATTACCGCCTTGAC	*arlS* DIG-labelled probe, rev	This work
MS62	GCCAGGTGTATACCAAATCAG	*hemB* DIG-labelled probe, fwd	This work
MS63	CGGTTAGCAGGGTCCATC	*hemB* DIG-labelled probe, rev	This work
MS64	CATTGCTGCTAAAGCACAAAG	*svrA* DIG-labelled probe, rev	This work
MS66	GCGCCGCCGAAG	*sae* T4 DIG-labelled probe, rev	This work
MS67	GAAGGATACGATGTGTACTGTGC	*yycFG* DIG-labelled probe, fwd	This work
MS68	CGTTTCGACCTCTACTCATGTTG	*yycFG* DIG-labelled probe, rev	This work
MS71	GCCACGATGCGTCC	pET24a(+) fwd, position 375, for sequencing	This work
MS74	GAATCCCCAAGCACCTAAAC	*isaA* DIG-labelled probe, rev	This work
MS75	GGCATCATCATTAGCAGTGG	*isaA* DIG-labelled probe, fwd	This work
RNAIII+	GTGATGGAAAATAGTTGATGAG	RNAIII DIG-labelled probe, fwd	(60)
RNAIII	GTGAATTTGTTCACTGTGTCG	RNAIII DIG-labelled probe, rev	(60)
SA*asp23*+	ATGACTGTAGATAACAATAAAGC	*asp23* DIG-labelled probe, fwd	(129)
SA*asp23*-	TTGTAAACCTTGTCTTTCTTGG	*asp23* DIG-labelled probe, rev	(129)
*sae*285U	CAAATTGAAGAAATGAGGAGTTA	*sae* T4 DIG-labelled probe, fwd	(363)
spa	TGAATTCGTAAACTAGGTGTAGG	*spa* DIG-labelled probe, fwd	(327)
spa for rev	CGGTACCAGGCTTGTTATTGTCTTCC	*spa* DIG-labelled probe, rev	(327)
sarR	CTTCTAATTCTGAAATCAG	*sarR* DIG-labelled probe, fwd	(327)
sarR for rev	GACATTAATGATTTAGTCAAC	*sarR* DIG-labelled probe, rev	(327)
Sa*sarA*+	AGGGAGGTTTTAAACATGGC	*sar* DIG-labelled probe, fwd	(60)
Sa*sarA*	CTCGACTCAATAATGATTCG	*sar* DIG-labelled probe, rev	(60)

8.2. Plasmids

TABLE 4. Description of plasmids used in this work.

Name	Description	Reference
pET24b(+)	expression plasmid, N-terminal T7-Tag® or C-terminal His_6-Tag®, T7 promoter, Km^r, low copy origin	Novagen
pMS10	pSR3, *femX* replaced by Strep-TagII® (*Asp*718-*Eco*RI[lost]), introduction of RBS and *Pst*I-*Sac*I-*Eco*RI, Tc^r	This work
pMS13	pMS10, *femA* (*Pst*I-*Eco*RI), Tc^r	This work
pMS14	pET24b(+) *glyS* (*Nhe*I-*Xho*I), Km^r	This work
pSR3	pAW8 *xylRPO femX* (*Hin*dIII-*Eco*RI), Tc^r	S. Rohrer, unpublished

A **B**



C

***Bgl*II** **T7 promoter** **lac operator** ***Xba*I**
AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAAT

T7-Tag
RBS ***Nde*I** ***Nhe*I** ***Bam*HI**
AATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**GCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCCG
MetAlaSerMetThrGlyGlyGlnGlnMetGlyArgAspPro

***Not*I** **His$_6$-Tag**
***Eco*RI*Sac*I** ***Sal*I** ***Hind*III** ***Xho*I**
AATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCAC**TGA**GATCCGGCTGCTAAC
AsnSerSerSerValAspLysLeuAlaAlaAlaLeuGluHisHisHisHisHisHisEnd

***Bpu*1102I** **T7...**
AAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAA

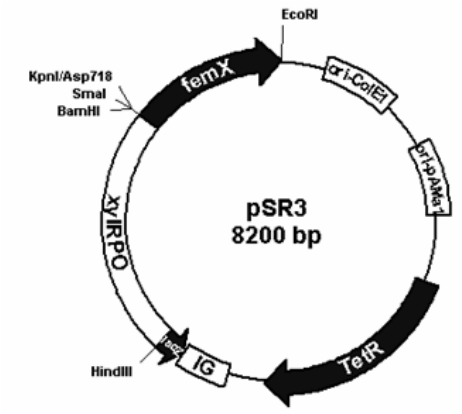
...terminator
CGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT

D

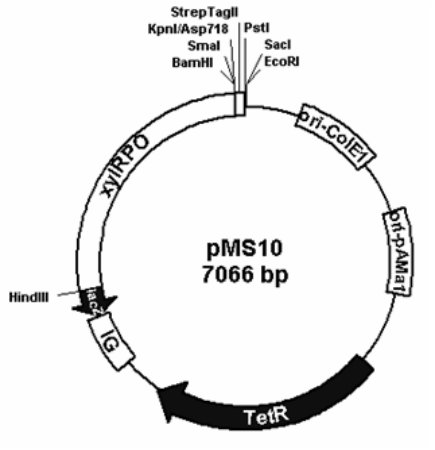
His$_6$-Tag
RBS ***Nde*I** ***Nhe*I** ***Xho*I**
GTTTAACTTTAAGAAGGAGATATACAT**ATG**GCTAGC-*glyS*-CTCGAGCACCACCACCACCACCAC**TGA**GATCCG
MetAlaSer-GlyS-LeuGluHisHisHisHisHisHisEnd

Fig. 48. pET24b(+) cloning vector (**A**) and its derivative pMS14 (**B**). **C** Multiple cloning site of pET24b(+). **D** pMS14 region encoding the recombinant protein GlyS-His$_6$.

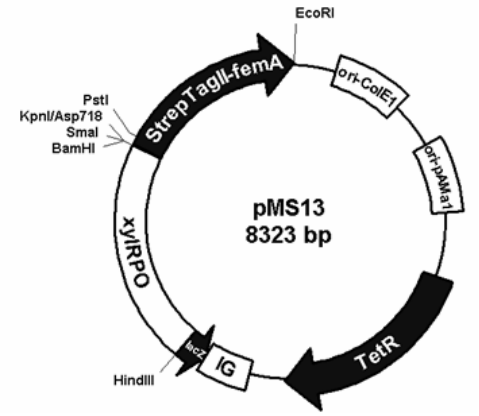
A



B



C



D

```
                          Strep-TagII
Asp718  RBS              ◄──────────►  PstI  SacI  EcoRI
GGTACCAGGAGGAATATTATGTGGTCACATCCACAATTTGAAAAACTGCAGGAGCTCGAATTCTAGT
                  MetTrpSerHisProGlnPheGluLysLeuGlnGluLeuGluPheEnd
```

E

```
                          Strep-TagII
Asp718  RBS              ◄──────────►  PstI        EcoRI
GGTACCAGGAGGAATATTATGTGGTCACATCCACAATTTGAAAAACTGCAG-femA-GAATTCTAGT
                  MetTrpSerHisProGlnPheGluLysLeuGln-FemA-GluPheEnd
```

Fig. 49. pSR3 (**A**) and its derivatives pMS10 (**B**) and pMS13 (**C**). **D** pMS10 region covering the Strep-TagII®. **E** pMS13 region encoding the recombinant protein Strep-TagII®–FemA.

8.3. Strains

TABLE 5. Strains used in this work.

Organism	Name	Description	Reference
E. coli			
	BL21	expression strain, DE3 (λ prophage carrying T7 RNA polymerase), *ompT lon* (protease mutations)	Novagen
	DH5α	cloning strain, blue-white selection, *recA1* Δ(*lacIZYA-argF*)	Invitrogen
	MS1	DH5α, pMS10 plasmid, Tc^r	This work
	MS10	DH5α, pMS13 plasmid, Tc^r	This work
	MS32	DH5α, pMS14 plasmid, Km^r	This work
S. aureus			
	III33	Newman *hemB*::*ermB*, SCV	(184)
	A22616/3	Clinical isolate, SCV of A22616/5, auxotrophic for menadione	(398)
	A22616/5	Clinical isolate, wt of A22616/3	(398)
	AS145	BB270 *femAB*::*tetK*, lysostaphin^r, Mc^s	(367)
	BB255	Essentially the same as NCTC8325, *rsbU*	(27)
	BB270	BB255 *mec*	(28)
	BB308	NCTC8325 *mec* Ω2003(*femA*::Tn*551*), Mc^s, Em^r	(28)
	BB1591	LS1 Δ*rsbUVWsigB*::*ermB*, Em^r	(272)
	GP266	RN4220 *rsbU*$^+$ *sigB1* (Am) -*tetL*, Tc^r	(37)
	GP268	BB255 (*rsbUVWsigB*)$^+$-*tetL*, Tc^r	(129)
	IK181	BB255 Δ*rsbUVWsigB*::*ermB*, Em^r	(214)
	IK184	Newman Δ*rsbUVWsigB*::*ermB*, Em^r	(214)
	LS1	Clinical osteomyelitis isolate	(51, 52)
	MB79	Newman P*asp23*::*luc*+-*tetL*, Tc^r	M. Bischoff
	MB138	BB255 *rsbW7* P*asp23*::pEC-P*asp23-luc*+, Em^r	(37)
	MS13	RN4220, pMS10 plasmid, Tc^r	This work
	MS14	RN4220, pMS13 plasmid, Tc^r	This work
	MS15	UK17, pMS13 plasmid, lysostaphin^s, Mc^r, Tc^r	This work
	MS16	UK17, pMS10 plasmid, lysostaphin^r, Mc^s, Tc^r	This work
	MS17	MB79 *hemB*::*ermB*, SCV, Am^{ir}, Em^r, Gm^{ir}, Tc^r	This work
	MS62	Newman *hemB*::*ermB sigB1* (Am), Em^r,Tc^r	This work
	Newman	Clinical isolate, high level of clumping factor, truncated fibronectin-binding proteins A and B	(93, 149)
	OM1a	Clinical isolate, wt of OM1b	C. von Eiff
	OM1b	Clinical isolate, SCV of OM1a, auxotrophic for menadione	C. von Eiff
	RN4220	NCTC8325-4, restriction-, modification+, prophage-free	(211)
	SR18	BB270 P*fmhB*::P*xyl*, Em^r	(325)
	UK17	BB270 *femA*	(207)
	$UT34_2$	NCTC8325 *mec* Ω2006 (*femB*::Tn *551*), Mc^s	(159)
S. simulans			
	22	Clinical isolate	(35)

8.4. Materials

8.4.1. Culture media

for 1 litre if not otherwise stated.

Agar/soft agar

15 g / 6 g Bacto Agar (Difco).

BHI

37 g Brain Heart Infusion (Difco); 200 g calf brain infusion, 250 g beef brain infusion, 10 g proteose peptone, 2 g dextrose, 5 g NaCl, 2.5 g Na_2PO_4.

LB

10 g Bacto-Tryptone (Difco), 5 g Bacto Yeast extract (Difco), 10 g NaCl, adjusted to pH 7 with NaOH, autoclaved.

MH

21 g Bacto Mueller-Hinton (Difco); 300 g beef infusion, 17.5 g caseine hydrolysate, 1.5 g starch.

Sheep blood agar

39 g Columbia Blood Agar Base No. 2 (Difco); 12 g Bacto Panton, 6 g Bacto Biton H, 3 g enzymatically digested animal tissue, 1 g starch, 5 g NaCl, 12 g Bacto agar, 50 ml sheep blood.

Skim milk

100 g skim milk (Difco).

Medium A (500 ml)

2.5 g yeast extract (0.5 %),10 g tryptone (2 %), 1 ml 5 M NaCl, 1.25 ml 1 M KCl (2.5 mM), 5 ml 1 M $MgCl_2$ (10 mM), 5 ml 1 M $MgSO_4$ (10 mM), adjusted to pH 7 with NaOH; autoclaved and stored at room temperature.

Medium B (250 ml)

12.5 ml 0.2 M PIPES pH 6.4 (10 mM), 13.75 ml 1 M $MnCl_2$ (55 mM), 3.75 ml 1 M $CaCl_2$ (15 mM), 62.5 ml 1 M KCl (250 mM), sterilized by filtration through a 0.2 μm filter and stored at RT.

8.4.2. Buffers

Solutions not listed here were made according to standard protocols (12).

1 M guanidine thiocyanate (100 ml)

11.816 g dissolved in dH_2O.

10 x Blocking reagent (100 ml)

10 g Blocking reagent (Roche) dissolved in 1 x maleic acid buffer, autoclaved.

5 x Maleic acid buffer (1 litre)

58.4 g Maleic acid (0.1 M), 43.83 g NaCl (0.15 M), adjusted to pH 7.5 with solid NaOH pellets, autoclaved.

Detection buffer pH 9.5 (1 litre)

12.11 g Tris (0.1 M), 5.844 g NaCl (0.1 M), pH has not to be adjusted, autoclaved.

5 x Blue (White) Wonder

4.4 M Urea, 2.7 % Nonidet P-40 (NP-40), 2.7 % β-mercaptoethanol, 0.16 M Tris, pH 6.8, 6.2 % SDS, 4.5 % glycerol and 0.01 % bromophenol blue. For White Wonder, the bromophenol blue was omitted to allow measurement of protein concentration, which was possible for protein concentrations ranging from 3 to 5 μg/μl as determined with BSA (despite the detergents present).

SMM buffer

0.5 M Sucrose, 0.02 M maleate, 0.02 M $MgCl_2$, pH 6.8, sterilized by filtration through a 0.22 μm filter and stored at RT.

Membrane buffer

0.1 M NaCl, 0.1 M Tris, 0.01 M $MgCl_2$, 0.2 mM PMSF (freshly added), pH 7.5.

8.4.3. Reagents

All chemicals and reagents were either purchased from Fluka, Sigma or Merck, unless stated otherwise.

Biofluor	Packard
Isopropyl β-D-thiogalactopyranoside (IPTG)	MBI Fermentas
TRIZol™	GibcoBRL
U-^{14}C-glycine	Amersham

8.4.4. Enzymes

All enzymes were purchased from Sigma or Roche, unless stated otherwise.

T4 DNA ligase	MBI Fermentas
Lysostaphin	AMBI

8.4.5. Molecular weight markers

1 kb DNA ladder	Gibco Life Technologies
1 kb Plus DNA ladder	Gibco Life Technologies
RNA ladder	New England Biolabs
Precision Plus Protein™ Standards, All Blue	BioRad
Prestained SDS-PAGE Standards, Broad Range	BioRad

8.4.6. Antibiotics

All antibiotics were purchased from Sigma or Roche. Etest® strips were from AB Biodisk.

The following concentrations were used for selection:

Ap: 100 μg/ml

Em: 20 μg/ml (in the case of selection of *hemB* mutants; 2.5 μg/ml)

Km: 20 μg/ml

Mc: 5-10 μg/ml

Tc: 10 μg/ml

8.4.7. Apparatus

Bio-imaging systems for	
documentation	CN-08 Photo-Print, Vilber Lourmat
documentation and quantification	ImageMaster VDS-CL and ImageQuant version 5.2, Amersham Pharmacia
Centrifugal filter devices, cutoff 10 kDa	Centricon, Millipore
Electroporator/cuvettes	GenePulser™, BioRad
Glass bead vials for reciprocating shaker	Lysing Matrix B, Bio101
Luminometer	Turner Designs TD-20/20 luminometer, Promega
Sterile filters, 0.45 μm and 0.22 μm pore size	Millex®, Millipore
PCR Cycler	Thermocycler Progene, Techne, Witec
PFGE	Chef-DR™ II Electrophoresis Cell, BioRad
Reciprocating shaker	Fast Prep FP120, Bio101
Research centrifuge	Sorvall® RC 5C Plus, Kendro
Scintillation counter	Liquid Scintillation Analyzer, TRI-CARB® 1900CA, Packard
Sequencer	ABI PRISM™ 310 Genetic Analyzer, Perkin- Elmer
Sonifier	B-12, Branson

Spectrophotometer	Ultrospec® 3000, Amersham Pharmacia
Table top centrifuges	5415D, Eppendorf Vaudaux
	5804R, Eppendorf Vaudaux
Ultracentrifuge	L-60, Beckman
UV Crosslinker (DNA/RNA)	UV-Stratalinker® 1800, Stratagene
Vacuum concentrator	SpeedVac Concentrator, Inotech

8.5. Molecular biology techniques

General molecular biology techniques were performed as described (12, 237).

8.5.1. Preparation of *E. coli* plasmid DNA

Plasmid minipreps were prepared from 4 ml of LB culture using the Nucleospin Plasmid mini Kit (Macherey-Nagel) according to the instructions of the manufacturer. For preparation of large amounts of plasmid DNA, midiprep kits (QIAGEN) were used. Plasmid DNA was dissolved in water. DNA concentration was determined by measuring the absorbance at 260/280 nm (1 OD_{260} unit = 50 μg/ml DNA_{ds}). Plasmid DNA was considered to be sufficiently clean if the ratio 260/280 nm was above 1.8. Plasmid DNA was stored at -20 °C.

8.5.2. Preparation of *S. aureus* genomic DNA

A 20 ml overnight culture was harvested and the pellet resuspended in 3 ml lysis mix (0.1 M Tris-HCl, pH 7.5; 0.1 M EDTA; 0.15 M NaCl) containing 0.05 mg lysostaphin and 0.25 mg lysozyme. The suspension was incubated at 37 °C until it became translucent and viscous. 0.3 ml of 5 % SDS in 50 % ethanol was added and the mixture was vortexed for 10'', then left on the benchtop for 5'. 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was added and the mixture was vortexed for 10'', then centrifuged at 20000 g for 10'. The aqueous phase was removed with a truncated Pasteur pipette, taking care not to disturb the proteinaceous interphase. The aqueous phase containing DNA was overlaid with 2 volumes of ice-cold 100 % ethanol and the DNA was spun out on a sterile glass rod. Spooled DNA was washed twice for 30'' in 70 % ethanol, briefly left to air-dry and dissolved in 0.5 ml TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA, pH 8). 1 µl of RNAse (1 mg/ml, DNAse free, Roche) was added and incubated at 37 °C for 1 h. DNA concentration was determined by measuring the absorbance at 260/280 nm (1 OD_{260} unit = 50 μg/ml DNA_{ds}). Genomic DNA was stored at 4 °C and was stable for more than 3 years.

8.5.3. Preparation of *S. aureus* plasmid DNA

Plasmid minipreps were prepared from at least 4 ml of LB overnight culture using the Nucleospin Plasmid mini Kit (Macherey-Nagel) with the addition of 5 µl of 10 mg/ml lysostaphin to the resuspension buffer 1 and incubation at 37 °C for about 10', or until the suspension became clear.

8.5.4. Preparation of *S. aureus* tRNA

50 ml of exponential phase culture (OD_{600} ≈ 1.5) was resuspended in 1 ml TRIZol® (GibcoBRL), transferred to glass bead vials and lysed for 40'' at a speed of 6.0 in the FastPrep beat-beater (Bio101). 0.2 ml chloroform was added. The mixture was vigorously shaken for 15'', and then left for 2' at RT before centrifuging for 2' at 13,400 g. The upper, aqueous phase was transferred to an Eppendorf tube, 0.5 ml isopropanol was added and shortly mixed before leaving for 10' at RT. Precipitated RNA was collected by centrifuging for 10' at 13000 g and 4 °C. The pellet was washed with 0.5 ml 70 % ethanol and centrifuged for 5' at 5400 x g and 4 °C. After briefly leaving to air-dry, RNA was dissolved in 0.5 ml dH_2O (DMPC treated) for 10' at 60 °C and shortly cooled at RT. Then, 0.325 ml 5 M NaCl was added, the mixture was vortexed and incubated 24 h on ice to precipitate high molecular weight RNA, which was collected by centrifuging for 30' at 16000 g and 4 °C. The opaque pellet was discarded and the supernatant mixed with 2 volumes 100 % ice-cold ethanol to precipitate small RNA at -20 °C overnight. The precipitate was collected by centrifuging for 5' at 2300 g and 4 °C. The pellet was washed with 0.5 ml 70% ethanol and centrifuged for 5' at 2300 g and 4 °C before drying up to 50' at 40 °C. tRNA was dissolved in 150 µl DMPC-treated dH_2O (if necessary for 10' at 60 °C). To allow correct folding of 3° structure tRNA was melted for 5' at 65 °C and slowly cooled to RT. tRNA concentration was determined at 260/280 nm (1 OD_{260} unit = 40 µg/ml RNA_{ss}). tRNA was stable for 8 months at – 20 °C.

8.5.5. Purification of DNA fragments

Restriction digests or impure PCR products were run on preparative 0.8 % agarose gels in 0.5 x Tris-borate EDTA (TBE) buffer according to (12). Bands were visualized on a long-wavelength ultraviolet light box for as short a time as possible to reduce DNA damage, excised using a scalpel and purified using the Nucleospin extract 2 in 1 kit (Macherey-Nagel) according to the manufacturer's specifications. Elution was usually done in 30 µl of elution

buffer. Yield and purity of the isolated fragments was judged by running them on a 0.8-2 % agarose gel and comparing the band intensity to a standard 1 kb DNA ladder (Gibco).

8.5.6. PCR

Preparative PCR reactions were generally carried out in 50 µl volumes. Usually, about 100 ng template DNA, 1/10 vol 10 x Pwo polymerase buffer (containing $MgCl_2$), 0.5 µM of each primer, 0.2 mM dNTPs, and 1 unit Pwo polymerase (Roche) was used. Alternatively, ProofStart polymerase (QIAGEN) was used. Reactions were run on a Techne Thermocycler (Witec) using the following conditions: initial denaturation at 95 °C for 4' followed by 25 cycles of 95 °C for 30'', annealing at 45-65 °C 30'', extension at 72 °C for 1-10', as the processivity of DNA polymerases is roughly 1-1.5 kb per minute. Annealing temperatures were usually chosen 2-3 °C below the theoretical melting temperature of the primer as calculated by the (2 AT+ 4 GC) rule. Usually, an initial 5 cycles using a touchdown protocol were inserted. In this method, the annealing temperature is reduced by 1 °C per cycle to ensure a more specific amplification of the desired product if the optimal annealing temperature of the primers has not been established. A final elongation at 72 °C for 8' was added.

For DIG-labelling of DNA probes the PCR DIG Probe Synthesis Kit (Roche) was used and the instructions of the manufacturer were followed. The ratio of unlabelled dNTPs (2 mM each) to Synthesis mix dNTPs (dATP, dCTP and dGTP 2 mM each; DIG-11-dUTP 0.7 mM) was 2, resulting in an end concentration of the labelled dUTP of 0.23 mM and of 1.33 mM dTTP. Annealing temperature was generally 50 °C, 30 cycles were run.

8.5.7. Cloning

8.5.7.1. Plasmid digestion

For preparative digestions, 3-10 µg plasmid DNA were digested with 10-30 U of enzyme in the appropriate 1 x restriction buffer for 2 hours. Alternatively, DNA was digested with 1-5 U of enzyme overnight. A list of enzymes that can be used in overnight digests can be found in the New England Biolabs catalog. Enzymes were from Roche.

8.5.7.2. Digestion of PCR fragments

PCR Fragments were routinely digested overnight, under the same conditions as for plasmids. Oligonucleotide primers that contained an overhang to incorporate a restriction site were designed with at least 3 extra nucleotides to ensure efficient cleavage by the restriction enzyme. Information about the restriction efficiency of different enzymes can be found in the back of the New England Biolabs catalog.

8.5.7.3. Dephosphorylation of 5' ends

Digestion with enzymes producing non-compatible ends but cutting at sites lying close together (which is usually the case when utilising an MCS) can produce linearised vectors cut just by one enzyme and consequently having compatible ends (running at practically the same height as the double-cut vector on a preparative agarose gel). 5' ends were therefore routinely dephosphorylated to inhibit self-ligation. Alkaline phosphatase from arctic shrimp was used (SAP, Roche or Promega). Dephosphorylation was done by incubating maximally 2 μg of linearised vector with 1 x SAP buffer and 1 U of SAP for 30' at 37 °C, followed by an inactivation step at 75 °C for 10'. After inactivation, an additional unit of SAP was added, incubation was continued at 37 °C for 30' and the reaction was terminated by incubation at 75 °C for 10'. In most cases, dephosphorylation was carried out directly after digestion in the restriction enzyme buffer since SAP is active in all restriction buffers.

8.5.7.4. Ligation of DNA fragments

DNA fragments were quantitated on agarose gels as described above. Generally, ligations were performed in 20 μl volumes using 20-50 ng of vector and insert:vector ratios of 3:1, 6:1 and nil:1 (negative control). The ratio is based on pmol molecules, assuming 1 bp = 649 Da. For convenience, the following formula can be used: ng insert = ((ng vector * insert size in kb) / vector size in kb) * insert:vector ratio. Ligation mixtures containing the appropriate insert:vector amount, 1 x ligation buffer and 0.5-1 μl of T4 DNA ligase (NEB) were incubated at 16 °C overnight. For electroporation, ligation reactions were precipitated using 1 μl of a 20 mg/ml solution of Blue Dextran (Sigma) in dH_2O, 2 μl of 5 M NaOAc pH 5.5, and 20 μl isopropanol. The reactions were precipitated on ice for 20', centrifuged at 4 °C and 16000 g for 20' in an Eppendorf table top centrifuge, washed twice with 70 % ethanol and left to air-dry at 37 °C for 10' or dried in a SpeedVac for no longer than 5'.

8.5.8. Sequencing

The ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) was used. This kit consists of an optimised *Thermus aquaticus* DNA polymerase for sequencing with less discrimination against dideoxynucleotides, eliminated 5'→ 3' nuclease activity and thermally stable inorganic pyrophosphatase. Included is a set of high-sensitivity dye terminators. Instructions of the manufacturer were followed for cycle sequencing (reaction volume 20 µl) and precipitation of products, with the exception of utilisation of 3 M NaOAc pH 5.5 instead of 4.5 and 100 % ethanol. The precipitate was dissolved in 20 µl formamide, heated to 90 °C for 2', vortexed, briefly centrifuged and kept on ice until sequencing. The ABI PRISM™ 310 Genetic Analyzer (Perkin- Elmer, 47cm x 50µm i.d. capillary) was used. Typically, 300-400 bases were sequenced with an injection of 60'' and reading for 120''.

8.5.9. Southern blot analyses

2 µg genomic DNA was digested with 10 U of restriction enzyme overnight and separated on a large (15 cm) 0.8 % agarose gel. The separation was documented using 1 kb DNA ladder (Gibco Life Technologies) for later size estimation of the hybridizing bands. The DNA was depurinated using 0.25 M HCl for 20' and denatured using 0.4 M NaOH for 30'. A capillary blotting method was applied for 3 h to transfer DNA fragments to a Biodyne A nylon membrane that had been prewetted in 0.4 M NaOH. The DNA was UV-crosslinked in a Stratalinker at the autocrosslink setting. The membrane was then briefly washed in dH_2O and prehybridised in 5 x SSC, 1 % Blocking reagent (Roche), 0.1 % N-lauroylsarcosine, 0.02 % SDS in maleic acid buffer. After prehybridisation for 30' at 55 °C, the heat-denatured, DIG-labelled PCR probe was added and hybridization was carried out overnight. The membrane was then washed twice for 15' at RT in 2 x SSC, 0.1 % SDS, and twice for 15' at 65 °C in 0.1 % SSC, 0.1 % SDS. The membrane was briefly rinsed in maleic acid buffer and incubated for 30' in 1 % blocking reagent in maleic acid buffer supplemented with 0.3 % Tween 20 (BioRad), and the alkaline phosphatase-coupled anti-DIG-Fab fragments were added at 1:20,000 dilution. After incubation for 1 h, the membrane was washed twice for 10' in maleic acid buffer/0.3 % Tween, then briefly rinsed in detection buffer. Detection was carried out using CDP-Star diluted 1:100 in detection buffer. Approximately 500 µl detection solution was distributed over a 12 x 15 cm membrane before sealing in plastic foil for detection.

8.5.10. Northern blot analyses

Total RNA was isolated as described previously (67), using the FastRNA kit and a Fastprep reciprocating shaker (Bio 101). For Northern blots, 5 to 10 µg of total RNA was loaded per lane, separated on a 1.5% agarose - 20 mM guanidine thiocyanate gel and transferred overnight onto a positively charged nylon membrane (Roche). The blots were hybridized with specific DIG-labelled DNA probes. Membranes were stripped by boiling twice in 0.1 SSC-0.5 % SDS for 30' and were reprobed up to four times.

8.5.11. mRNA half-life determination

Half-life of potentially more stable transcripts was determined at that time point of growth where the supposedly less stable transcripts began to disappear as observed by Northern blot analyses (see text). Bacteria grown for 5 hours in LB were supplemented with 300 mg/ml rifampin and samples were taken at three minute intervals. Total mRNA was isolated and analysed by Northern blots, as described above. Band intensities were quantified densitometrically with an ImageMaster VDS-CL using the ImageQuant version 5.2 (Amersham Pharmacia Biotech). Values were corrected against the background and normalized. Half-life of mRNA was determined from regression lines obtained by plotting mean values of at least two independent experiments against time on a semi-logarithmic graph. The regression line was calibrated to intercept with the initial amount of transcripts, which was set to 100.

8.5.12. Preparation of protein extracts

Total protein was isolated by resuspending cells in PBS, pH 7.4, at an OD_{600} corresponding to 2/ml. Glass beads (Sigma) were added up to the meniscus and the cells were lysed for 40'' at a speed of 6 in a Fastprep reciprocating shaker (Bio 101). After briefly cooling on ice, 0.1 ml PBS and 50 µl 5 x Blue Wonder were added. The mixture was vortexed and boiled for 5', shortly centrifuged and the supernatant transferred to a fresh tube. For cytoplasmic fractions, pelleted bacteria were resuspended in 0.07 M phosphate buffer, pH 6.8, containing lysozyme, lysostaphin and DNase, each at 0.018 mg/ml, as well as 2 mM phenylmethylsulphonylfluoride (PMSF). After incubating at 37 °C for 30' the solution was sonicated thrice for 10'' with a Branson B-12 sonifier on ice. Cell debris were pelleted by centrifuging for 20' at 14000 rpm and 4 °C. Protein concentration of the supernatant was determined by the Bradford method (49) with BSA as a standard.

Fractionated extracts were obtained by resuspending cells in half the original volume of SMM buffer, containing lysostaphin 0.1 mg/ml. Cells were lysed at 37 °C for 10' and centrifuged for 4' at 30000 g. The supernatant contained the cell wall fraction and was precipitated with TCA as described below. The pellet, containing protoplasts, was resuspended in a quarter of the original volume of membrane buffer and subjected to a freeze-thaw cycle in dry ice/ethanol. Membranes were collected by centrifuging for 1 h at 50000 g in an ultracentrifuge. The pellet was resuspended in phosphate buffer, the supernatant containing the cytoplasmic proteins was TCA precipitated as follows.

Supernatants were precipitated by addition of TCA up to an end-concentration of 10 %. This mixture was left on ice for 30', then centrifuged for 15' at 30000 g. The pellet was washed with ice-cold acetone, followed by centrifugation for 5' at 30000 g. After drying the precipitate, proteins were dissolved in 5 x Blue Wonder. If the buffer turned yellow because the sample was too acidic, 1 M Tris, pH 8, was added until neutralisation (blue colour).

8.5.13. Overexpression and purification of His-tagged proteins

Recombinant protein bearing a C-terminal hexahistidine tag was expressed in *E. coli* BL21(DE3) cells using the pET system (Novagen). BL21(DE3) cells are all-purpose strains for high-level protein expression and easy induction. BL21(DE3)pLysS cells provide tighter control for expression of toxic proteins. In addition, BL21 cells can be used for non-T7 RNA polymerase protein expression systems. All BL21 strains are deficient in the OmpT and Lon proteases, which may interfere with isolation of intact recombinant proteins. The pET vector series contains a phage T7 promoter, while the DE3 determinant of the BL21 strains encodes a T7 RNA polymerase under the control of the *lac* promoter. Induction of the T7 polymerase using IPTG allows controlled expression of high amounts of recombinant protein (148). GlyS was cloned in *E. coli* DH5α into pET24b(+) (Novagen) using PCR primers tailed with *Nhe*I and *Xho*I sites such that the hexahistidine tag was fused in frame to the C-terminus. This was done to ensure the purification of full-length protein only.

Plasmids encoding FemA-, FemB- or FemX-His_6 (which had been previously constructed (323)) and GlyS-His_6 were freshly transformed into BL21(DE3) electrocompetent cells. Transformed BL21 cells were not stored as frozen stocks; instead, they were electroporated freshly every 4-5 weeks. In each case one transformant was used to inoculate 10 ml LB + 20 μg/ml Km pre-cultures, grown overnight at 37 °C. 500 ml main cultures were started with an OD_{600} of 0.01 and incubated at 30 °C to favour proper folding of protein and decrease the risk of inclusion bodies. Induction with 0.5 mM IPTG was started when the cultures had reached an OD_{600} of 0.6. The cells were harvested after 90' and stored at -20 °C. Cells were lysed in 20 ml of lysis buffer (50 mM Na-Phosphate pH 8.0; 300 mM NaCl;

10 mM imidazole; 1 mg/ml lysozyme; 0.1 mg/ml DNAse; 0.01 mg/ml RNAse) for 30' on ice. An aliquot of this total lysate was taken for SDS-PAGE analysis. The lysate was sonicated on ice; 3 bursts of 10'' were applied. The lysates were then centrifuged at 20'000 g for 20' to yield cleared lysate. An aliquot of the cleared lysate was taken for SDS-PAGE to observe the ratio of soluble versus insoluble recombinant protein.

The cleared lysate was incubated with 1 ml Ni-NTA-agarose resin (QIAGEN) for 1 h, on an overhead shaker, at 4 °C. The Ni-NTA resin has a binding capacity of 5 mg protein per ml of column volume. A mini-column method was used, for which small columns with a porous frit (Quickspin, Roche) had been welded to a 10 ml plastic syringe. A small amount of the post-incubation supernatant ("flow-through") was retained for SDS-PAGE analysis. The resin was washed with 5 ml of wash buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). The bound protein was eluted with 3 x 0.5 ml of elution buffer (50 mM Na-phosphate, pH 8.0, 100 mM NaCl, 200 mM imidazole). To concentrate the eluted protein the eluates were centrifuged in Centricon centrifugal filter devices with a cutoff of 10 kDa (Millipore) at max. 5000 g up to 5 x for 20'. 0.5 ml glycerol was added as an antifreeze in order to store the enzymes at – 20 °C, where they remained stable and active for at least one year.

The recombinant proteins obtained with this procedure were highly, but not absolutely pure. Contaminating bands with affinity to the Ni-NTA-agarose resin were not removed (Fig. 30). These proteins are estimated to make up less than 10 % of the total protein obtained. Since GlyS and the Fem factors eluted from the column at imidazole concentrations higher than about 20 mM, a more stringent wash procedure could not be applied.

8.5.14. SDS-PAGE and Western blot analyses

Standard protocols for discontinuous SDS-polyacrylamide gel electrophoresis (219), electroblotting (379), and staining (12) were used. Precision Plus Protein All Blue Standards (Bio-Rad) was used as a molecular size marker. Antibodies were generally incubated in PBS, 0.5 % skim milk powder, 0.3 % Tween 20 (BioRad) at appropriate dilutions ranging from 1:1000 to 1:10000 for 1 h, followed by washing in PBS, 0.3 % Tween for 3 x 5'. Detection of horse radish peroxidase (HRP)-conjugated antibodies was with ECL (Amersham) or Super Signal West Pico (Pierce) reagents according to the recommendations of the manufacturers. For SigB detection, blocked membranes were preincubated with 40 μg/ml human IgG (Calbiochem) to saturate protein A and thereby prevent cross-reactivity of antigen-purified, rabbit antibodies against SigB.

8.5.15. Protein sequencing

Recommendations from BioRad for Immun-Blot® and Sequi-Blot® were followed. An estimated 6 μg of recombinant SigB-His_6 protein isolated from BL21 was gel-purified by SDS - 10 % PAGE and transferred onto a PVDF-membrane (BioRad). Transfer efficiency was determined by comparing stained gel and membrane (33.3%). The band of interest was excised with a scalpel, thoroughly rinsed with 0.45 μm filtered dH_2O and air-dried. The first five amino acids were determined by Edman sequencing performed at the Protein Service Laboratory of ETH, Zürich (http://www.biol.ethz.ch/dienstleistungen/Protein_Service_Lab).

8.5.16. Antibody production

An estimated 0.86 mg of recombinant SigB-His_6 protein isolated from BL21 was gel-purified by SDS - 10 % PAGE, Coomassie-stained and excised with a scalpel. Gel-homogenisation and chicken polyclonal antibody-production was performed by Davids Biotechnology, Regensburg, according to the DabioPrepI protocol (www.dabio.de).

8.5.17. Antigen-purification of antibodies

Recombinant, His_6-tagged target protein (1.5 μg – 1 mg) isolated from BL21 was gel-purified by SDS-PAGE (10 – 12%) and blotted onto a nitrocellulose membrane (Hybond, Amersham Pharmacia). The membrane was stained with Amido Black and the desired band was excised with a scalpel. After blocking with 5 % skim milk/PBS for 30' at RT, the membrane was briefly washed twice with PBS. Crude antibody-containing solution was added and either incubated for 2 hours at RT or overnight at 4 °C. After washing twice for 15' with PBS, bound antibodies were eluted with 0.1 M glycine, pH 2.2, and immediately neutralised with 1 M Tris-HCl, pH 7.5. 15 mM Na-azide was added as a preservative. Optionally, BSA was added to a concentration of 0.1 mg/ml for stabilising the antibodies.

8.5.18. Luciferase assay

Bacteria were harvested by centrifugation and the pellet was flash-frozen in liquid nitrogen. Cells were resuspended to an OD_{600} of 10/ml, corresponding to 10^9 cells/ml, in 0.7 M phosphate buffer, pH 6.8, containing lysozyme and lysostaphin, each at 0.036 mg/ml. After shaking for 10' at 37 °C, 10 μl supernatant of lysed cells was mixed with an equal

volume of luciferase assay substrate (Promega) and luminescence was measured for 15'' after a delay of 3'' on a Turner Designs TD-20/20 luminometer (Promega). According to the manufacturer's information the luciferase assay substrate contains excess ATP; addition of 2 mM ATP did not increase signals. Protein concentration of the supernatant was determined by the Bradford method (49) with BSA as a standard.

8.5.19. GlyS activity assay

GlyS-His_6 activity was tested in a 50 µl reaction mixture containing 9 µg enzyme (negative control dH_2O), 10 µg total tRNA, 4 mM ATP, 0.8 mM DTT and 0.5 nmol/2µl U-^{14}C-glycine (Amersham, 50 µCi/ml) in 50 mM Tris-HCl , pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl. The mixture was incubated for 1 h at 30 °C and then applied to 1 cm^2 of Whatman filter paper No.1. 0.3 ml of ice-cold 10 % TCA was added and mixtures were briefly kept on ice to precipitate tRNA. Afterwards filters were washed twice with 0.3 ml ice-cold 5 % TCA and once with 0.3 ml ice-cold 100 % ethanol. Filters were briefly air-dried before transferring into a scintillation-counter vial. 5 ml biofluor (Packard) was added, incorporation of radioactivity determined with a TriCarb scintillation counter. Standard curves were determined in parallel by dilution series of U-^{14}C-glycine in dH_2O.

8.5.20. In vitro cell wall synthesis assay

Experimental procedures for the establishment, and experiments performed with the *in vitro* assay for peptidoglycan synthesis are described in (349).

8.6. Bacteriological methods

8.6.1. Stock cultures

Stock cultures were maintained at –80 °C in skim milk medium.

8.6.2. Preparation of competent cells and transformation

Chemically competent cells (173)

E. coli DH5α cells were streaked out on LB plates from a skim milk stock and incubated over night at 37 °C. 5-10 single colonies were transferred into 250 ml Medium A and incubated at RT (or less) with shaking until they reached an OD_{600} of 0.5 (usually 2-3 days). Cells were incubated on ice for 10' and pelleted at 4000 g for 10' and resuspended in 80 ml ice-cold medium B, followed by 10' on ice. Cells were pelleted again, resuspended in 20 ml ice-cold medium B and slowly stirred on ice. While stirring, 1.4 ml dimethyl sulfoxide (DMSO) was added dropwise and cells were incubated on ice for another 10'. 400 µl aliquots were flash-frozen in liquid nitrogen and stored at -80 °C.

For transformation, cells were thawed on ice, divided into 100 µl aliquots and 10 µl ligation mixture or 1-100 ng supercoiled plasmid DNA was added. The transformation mix was incubated on ice for 30', heat-shocked at 42 °C for 90'', incubated on ice for 3', and 400 µl LB medium was added. Cells were grown at 37 °C for 30' – 1 h. After a short centrifugation step approximately half of the supernatant was discarded, cells were resuspended in the remaining 200 µl, spread on selective plates and incubated at 37 °C overnight. This protocol yields approximately 10^7 to 10^8 transformants per µg plasmid DNA.

Electrocompetent *E. coli* cells

A 500 ml culture of *E. coli* was grown to an OD_{600} of 0.7 and harvested at 10000 g for 10'. The cell pellet was resuspended in 250 ml of ice-cold dH_2O and centrifuged again at the above settings. The procedure was repeated with 0.25 and 0.125 volumes of the original culture. The pellet was resuspended in 20 ml of ice-cold 10 % glycerol, centrifuged, resuspended in 2 ml of ice-cold 10 % glycerol and finally dispensed in 120 µl aliquots, which were subsequently flash-frozen in liquid nitrogen.

For electroporation, 60 µl of cells were mixed with 1 µl of precipitated ligation mixture or approximately 100 ng plasmid miniprep DNA and incubated on ice for 1'. Electroporation

was carried out with a BioRad GenePulser using 0.2 cm electrode gap cuvettes at 25 µF, 200 Ω, 2.5 kV. This protocol yields up to 10^9 transformants per µg of plasmid DNA.

Preparation of electrocompetent *S. aureus* cells

A 500 ml culture was grown to an OD_{600} of 0.7 and harvested at 10000 g for 10' at 4 °C. The cell pellet was resuspended in 250 ml (0.5 volume of the original culture) of ice-cold 0.5 M sucrose and centrifuged again at the above settings. The procedure was repeated with 0.25, 0.125 and 0.0625 volumes of the original culture, with an incubation step of 2-3 hours at the third wash cycle. The pellet was then resuspended in 20 ml of ice-cold 10 % glycerol. After the following centrifugation step, the cells were resuspended in 2 ml of ice-cold 10 % glycerol, dispensed in 120 µl aliquots and flash-frozen in liquid nitrogen.

For electroporation, 60 µl of cells were mixed with 1 µl of plasmid miniprep DNA and incubated on ice for 30'. Electroporation was done in 0.1 cm electrode gap cuvettes at 25 µF, 100 Ω, 1.5 kV. Immediately after electroporation, 1 ml of cold BHI broth was added and cells incubated for 90' at 37 °C before selective plating.

8.6.3. Phage transduction

For the production of phage lysate, $CaCl_2$ was added to 20 ml overnight cultures of the donor strain in LB to an end concentration of 5 mM. 300 µl aliquots of culture were mixed with 100 µl of serial dilutions (10^{-1} to 10^{-5}) of phage lysate (80α or 85α) and incubated at RT for 15'. 4 ml of soft agar (approx. 55 °C) containing 5 mM $CaCl_2$ was added and the mixtures plated on sheep blood agar plates. After overnight incubation at 37 °C, a plate that had a confluent lysis pattern, but a brown colour due to hemolysis to indicate growth of the cells was selected. The soft agar layer was scraped off into 4 ml of LB with 5 mM $CaCl_2$ and centrifuged at 13000 g for 10' at 4 °C. The supernatant was sterile filtered through a 0.22 µm filter.

For phage transduction, the recipient strain was grown overnight in LB and $CaCl_2$ added to 5 mM. 300 µl aliquots were mixed with 100 µl of serial dilutions of transducing phage lysate and incubated at RT for 15'. 4 ml of soft agar (approx. 55 °C) containing 20 mM trisodium citrate was added and the mixtures plated on selective media. Colonies appeared after 24-72 hours.

8.6.4. Susceptibility testing

Etests

The MIC of standard antibiotics was measured by Etest (AB Biodisk, Solna, Sweden) on sheep blood or Müller-Hinton agar. A McFarland 0.5 (approximately 10^8 cfu/ml) suspension of the strains was swabbed on an agar plate in three directions and briefly left to dry. The test strip was then applied and the plates were incubated for at least 24 h at 37 °C. In the case of *hemB* mutants, the MICs were first read after 24 h, the last reading was after 72 hours.

Gradient plates

Gradient plates were prepared in rectangular inoculum dishes (Dynatech) using 40 ml of LB bottom agar containing the required maximal amount of antimicrobial, and 40 ml of LB top agar. Plates were dried for 2 h before application of a bacterial suspension adjusted to McFarland 0.5 with a cotton swab along the gradient. Growth was assessed after 24 h incubation.

9 APPENDIX

9.1. Sublocalisation of Fem-Factors

The X-ray crystal structure of two Fem factors revealed that they do not contain membrane-spanning domains (26, 34). However, it was plausible to assume that these factors are somehow membrane-associated, as their substrates are lipid intermediates (156, 193, 249). FemA had previously been found in the cytoplasma (182). In our group, M. Tschierske detected FemB both in the cytoplasmic and the membrane fraction.
To complete data, it was planned to detect FemX with chicken antibodies in fractionated cell preparations. Chicken antibodies (IgY) have the advantage to be bound less by protein A, which usually cross-reacts with the constant region of IgGs.

A different approach allowing more convenient determination of *in situ* expression and localisation of the Fem factors, was followed in parallel. In a first attempt, a StrepTag II® was fused to the N-terminus of FemA, which, as deduced from the X-ray structure, is not buried in the protein and thus theoretically suitable for labelling (Fig. 50). The StrepTag II® is an octapeptide mimicking biotin and therefore recognised by streptavidin conjugates (235, 348).

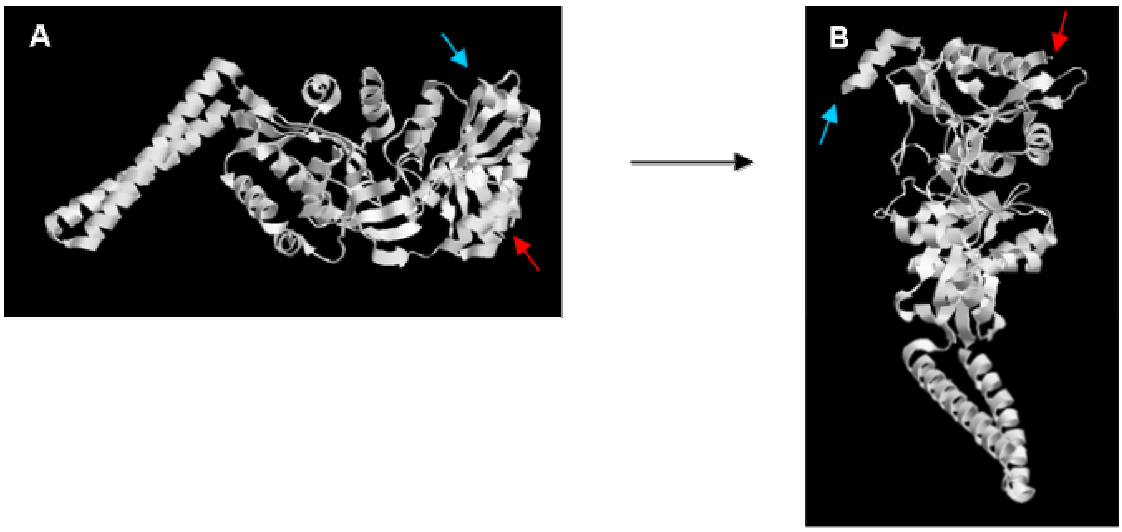


Fig. 50. A Ribbon structure of FemA positioned as in Fig. 23. **B** FemA, after tilting in the plane -90 ° and rotating -90 ° out of the plane; the coiled-coil domain pointing to the front. The N-term is indicated by a red, the C-term by a blue arrow, respectively. Image created with RasTop (version 2.6) from Protein Data Bank entry 1LRZ, determined by (26).

FemX

IgYs produced against recombinant FemX-His_6 were collected at different time points of immunisation and tested for specific recognition of FemX-His_6 (performed by S. Rohrer, M. Peter). The best fractions were pooled and antigen-purified. As determined by comparison with recombinant FemA-His_6 and FemB-His_6, the antibodies recognised only FemX-His_6. When comparing cell membrane fractions of strains BB308 (*femA*), $UT34_2$ (*femB*), AS145 (*femAB*) and SR18 (conditional *femX* under the control of the *xyl* operator) grown in the presence of xylose (enhanced FemX production) or glucose (repressed FemX production) (325), FemX was detected strongest in SR18 grown with xylose. A distinct, but weaker band was visible also under repressed conditions, indicating that repression had not been complete. Fractionated cell preparations from BB270 (wt) showed that, similar as found for FemB, FemX could be isolated both from the cytoplasmic as well as membranous compartment. Apparently, with the methods used, the soluble proteins FemB and FemX were not completely separated from the membrane fraction, possibly because they associate with the membrane, e.g. by binding to their substrate or to membrane proteins.

FemA

For expression of the StrepTag II®-FemA fusion protein, a shuttle vector containing the above mentioned *xyl* operator region, was used (pMS13). Without induction, sufficient functional fusion protein as to restore methicillin resistance was produced in the *mecA* containing strain UK17 (*femA*) when pMS13 was transduced into it. StrepTag II®-FemA-linked complementation was confirmed by allowing plasmid loss under non-selective growth conditions, whereby methicillin resistance (conferred by the functional FemA) as well as tetracycline resistance (mediated by the plasmid backbone) was lost. Therefore, the tagged FemA fusion protein was functional and no other unwanted chromosomal modification was responsible for complementation. Unfortunately, Western blot analyses with Streptavidin-alkaline phosphatase (AP) comparing protein extracts from UK17, respectively UK17 containing pMS13, did not identify a specific band of the expected size. Possibly, protein levels were too low, and StrepTag II®-FemA fusion protein needs to be induced. However, since the same regulatory element as for FemX was used in the same genetic background BB270 (see above), levels should be comparable. It could be that, despite appearances from the crystal structure of FemA, the N-term and the relatively small StrepTag II® were not accessible for Streptavidin-AP. Therefore, a linker region might be added and/or tagging at the C-term attempted. To reduce the background, blocking with egg white avidin, which is not recognised by Streptavidin, but can bind to biotinylated host proteins, should be tried.

9.2. Altered protein levels in the *hemB* mutant

The pattern of cytoplasmic protein fractions in the *hemB* mutant MS17 was strikingly different compared to wild type MB79 (Fig. 40D). Besides a prominent band at about 48 kDa, seen in both strains, strong bands appeared at approximately 40 and 85 kDa in MS17. Two nearby migrating bands of 40 and 38 kDa most probably correspond to ornithine transcarbomylase (39 kDa) and lactate dehydrogenase (37 kDa), shown to be strongly up-regulated in SCVs (205, 247). The band of 85 kDa might correspond to pyruvate-formate-lyase (85 kDa) or the ATP-dependent Clp protease chain ClpL (81 kDa), cytoplasmic proteins found to be produced in large amounts in a COL *hemB* mutant (205).

9.3. Monitoring of strains MB79 and MS17 during 7 days

Bacteria grown in batch culture for several days pass through five distinct phases. During exponential phase, which follows an initial lag phase, the population grows rapidly. Carbohydrates initially serve as the primary carbon and energy source but are quickly spent, and other nutrients, including peptides, amino acids, nucleic acids, nucleotides, and fatty acids, are utilized to sustain growth. As these remaining nutrients are depleted, the population enters stationary phase, during which little change is observed in the number of viable cells in the culture for 2 to 3 days. As the culture enters death phase, cell viability abruptly declines and approximately 99% of the cells die. Finally, during long-term stationary phase, there is a long period of slow decline in the remaining viable cell population (103).

In order to characterise the two strains MB79 and MS17 in terms of fitness and survival capacity over prolonged growth, as well as to find out whether the *agr* locus can be activated in the *hemB* mutant irrespective of low cell densities, bacteria were monitored during 7 days. Growth, colony forming units (cfu), luciferase activity, *sigB* and *agr* transcription were analysed. Contrary to the above described study of Newman and its *hemB* mutant, bacterial cultures were started with unwashed overnight cells and an OD_{600} of 0.01.

Gene expression

RNA from wild-type cells grown for more than three days seemed to degrade more easily, as determined from analytical agarose gels; RNA from the *hemB* mutant looked more stable (Fig. 51). Therefore, RNA from days 2-6 was blotted onto membranes by the use of a BIO DOT SF apparatus (BioRad). This method does not allow differentiating between overlapping transcripts of one gene, but made it possible to detect mRNA even in its

degraded form. To determine the amount of RNA blotted, aliquots were hybridised with DIG-labelled 16S rRNA probes.

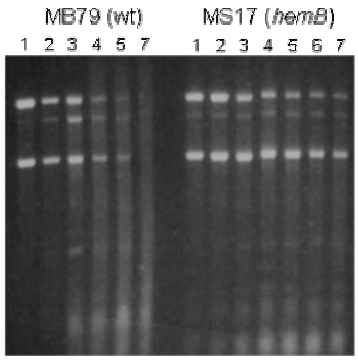


Fig. 51. RNA integrity of strains MB79 (wt) and MS17(*hemB*). RNA sampled over seven days was isolated as described in Materials and Methods. 3 μg were separated on a 1.5 % agarose-MOPS gel. The MB79 sample of day 6 is missing.

Interestingly, transcription of *agr* and *sigB* was strongly reduced in MB79 on day 4 (81 h); in MS17 it was slightly reduced on day 3 (61 h); thereafter transcription increased again (Fig. 52). Luciferase activity decreased rapidly in MB79 to levels similar as found in MS17, in agreement with the trend observed during stationary phase described earlier for MB79 (Fig. 40).

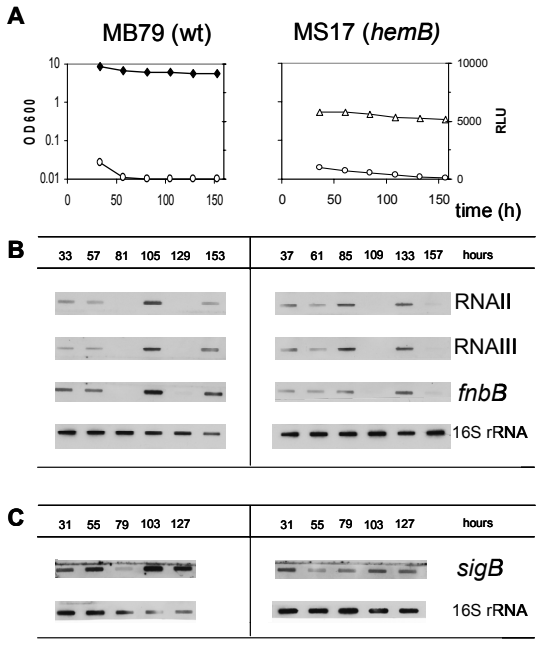


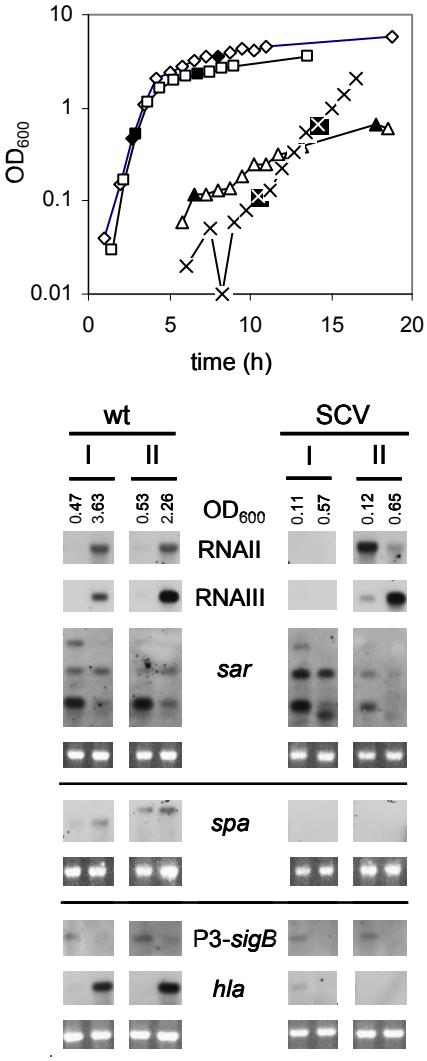
Fig. 52. Gene expression of strains MB79 (wt) and MS17 (*hemB*) during seven days. **A** Growth as determined by measuring OD_{600} (MB79, diamonds; MS17, triangles) and luciferase activity (circles). A different luciferase substrate batch had been used than in Fig. 40, giving maximal RLUs during the first day (not shown) of 9179 (MB79) and 2603 (MS17). **B** *agr* and *fnbB* expression. **C** *sigB* expression, stemming from another growth curve which was sampled differently and only monitored during 127 hours. Samples were taken every 24 hours, prepared as described in Materials and Methods and transferred onto a nylon membrane by slot blotting. Bacteria were grown as described in Fig. 36A and B; data shown here under A and B are the same and originate from that growth curve.

A fluctuating on/off gene expression might be explained by adaptation of a few bacteria during the stationary phase, where depletion of nutrients and pH increase of the medium are the major adverse factors. After reduction of the initial bacterial population in death phase, a new generation of bacteria might then be able to grow up, going through an entire growth cycle again with its corresponding characteristic gene expression. Why luciferase activity does not resume, remains to be determined.

9.4. Gene expression in clinical SCV isolates

Gene expression of the global regulators *agr*, *sar*, *sigB* and the virulence determinants *spa* and *hla* was analysed in two clinical menadione-auxotrophic SCVs and compared to their isogenic wild-type parents. Bacterial cultures were started with unwashed overnight cells and an OD_{600} of 0.01, explaining the lag of approximately 5 hours before SCVs started growing. In the wild-type strains, representative samples of exponential and post-exponential phase were taken. For SCVs, a sample at OD_{600} = 0.1 and at the exit of exponential phase was taken. Therefore, the expression profiles could not be compared between parent and SCVs, but roughly reflected the time course of gene expression.

Transcription of the analysed genes corresponded in the wild-type strain to what was expected; *agr*, *spa* and *hla* transcription started after the exponential phase (Fig. 53).



spa transcripts differed in size in these two genetic backgrounds, exemplifying the heterogeneity of this gene (53). All *sar* transcripts were present early in growth (corresponding to hour 2 of MB79), thereafter *sarB* and *sarA* transcripts decreased. Only the 1.6 kb P3-*sigB* mRNA of the *sigB* operon was analysed, which in both parent strains was stronger in exponential phase. It is well possible that this transcript strongly fluctuates, for which the second time point might have missed the increase observed in MB79.

Fig. 53. Gene expression in two clinical SCV isolates and their isogenic wild-type parents. RNA sampling for wild-type bacteria (I: A22616/5, diamonds; II: OM1a, squares) was performed at mid- and post-exponential growth phase, whereas SCVs (I: A22616/3, triangles; II: OM1b, crosses) were sampled at early and post-exponential phase (indicated by dark fillings). Ethidium-bromide stained 16S rRNA is shown as an indication of RNA loading.

In the SCVs partly varying profiles were observed. In both strains *spa* and *hla* were strongly reduced or absent. While *agr* was not at all transcribed in the SCV OM1b, similar to the *hemB* mutant MS17, in the SCV A22616/3 *agr* was expressed. Surprisingly, RNAII was present very early in growth, triggering RNAIII transcription earlier than in the parent strain A22616/5. In both SCVs, the 1.6 kb P3-*sigB* mRNA was weaker than in the corresponding parent strains and reduced to the first time point of sampling. However, as seen for the stationary phase of MS17, low *sigB* transcription does not necessarily equal low SigB activity. At least in the SCV A22616/3, expression of the SigB-dependent *sarC* transcript seemed to be induced, suggesting that SigB might play a role in a subgroup of clinical SCVs. As seen for OM1b, reversion to wild-type phenotype can occur during cultivation of clinical SCVs. While the expression profile of OM1b still corresponded at the two time points analysed to the one of an SCV, the culture might have already been a mixture of SCVs and wild-type strains.

9.5. Abbreviations

aa	amino acid(s)
AIP	auto-inducing peptide
Am	amber (UAG stop codon)
Ap	ampicillin
AP	alkaline phosphatase
ATCC	American Type Culture Collection, Manassas, Virginia
BHI	brain heart infusion
bp	base pairs
cfu	colony forming units
DIG	digoxigenin
ECM	extracellular matrix
$Em^{r/s}$	erythromycin resistant/sensitive
fwd	forward primer
His_6-Tag	hexahistidine tag
HRP	horse radish peroxidase
ICAM	intercellular adhesion molecules
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranoside
ir	intermediate resistant
IS	insertion sequence
$Km^{r/s}$	kanamycin resistant/sensitive
LB	Luria-Bertani (medium)
$Mc^{r/s}$	methicillin resistant/sensitive
MCS	multiple cloning site
mec	methicillin resistance determinant
MH	Müller-Hinton
MIC	minimal inhibitory concentration
MLS	macrolide, lincosamide, streptogramin
MOPS	morpholinepropanesulfonic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
Mw	molecular weight
NCCLS	National Council on Clinical Laboratory Standards (USA)
NCTC	National Collection of Type Cultures
Ni-NTA	Ni_2^+-nitrilotetraacetate

nt	nucleotides
OD	optical density
ORF	open reading frame
ori	origin of replication
P	promoter
PFGE	pulsed field gel electrophoresis
PFT	platelet-fibrin thrombi
PMA	phosphomolybdic acid
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulfonylfluoride
PVDF	polyvinylidene difluoride
RBS	ribosomal binding site
rev	reverse primer
RNAP	RNA polymerase core enzyme
rpm	revolutions per minute
RT	room temperature
SCC*mec*	staphylococcal cassette chromosome *mec*
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SXT	trimethoprim-sulfamethoxazole
TEM	transmission electron microscopy
TCA	trichloroacetic acid
$Tc^{r/s}$	tetracycline resistant/sensitive
Tn	transposon
TLC	thin layer chromatography
TNF	tumor necrosis factor
TSST	toxic shock syndrome toxin
U	unit of enzyme activity
VCAM	vascular-cell adhesion molecules
wt	wild type
xylRPO	xylose regulon

9.6. Literature

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9.8. Curriculum vitae

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Education

2001 – 2005	Doctoral work at the Institute of Medical Microbiology, University of Zürich
2000	Graduation at the Institute of Molecular Biology, University of Zürich
1993 – 2000	Study of Biology at the University of Zürich
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Publications

Entenza, JM, Moreillon P, Senn MM, Kormanec J, Dunman PM, Berger-Bächi B, Projan SJ, Bischoff M. 2005. Role of σ^B in the expression of *Staphylococcus aureus* cell-wall adhesins ClfA and FnbA and contribution to infectivity in a rat model of experimental endocarditis. Infect. Immun. 73:990-998.

Schneider T, Senn MM, Berger-Bächi B, Tossi A, Sahl HG, Wiedemann I. 2004. *In vitro* assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly_5) of *Staphylococcus aureus*. Mol. Microbiol. 53:675-85.

Nair SP, Bischoff M, Senn MM, Berger-Bächi B. 2003. The σ^B regulon influences internalization of *Staphylococcus aureus* by osteoblasts. Infect. Immun. 71:4167-70.

In preparation:

Senn MM, Bischoff M, von Eiff C, Berger-Bächi B. σ^B activity in a *Staphylococcus aureus hemB* mutant.

Senn MM, Giachino P, Homerova D, Steinhuber A, Strassner J, Kormanec J, Flückiger U, Berger-Bächi B, Bischoff M. Molecular analysis and organization of the σ^B operon in *Staphylococcus aureus*.

Conference presentations

2004 ISSSI, Charleston SC, USA.
Poster presentation: New insights into the transcription profile of a *Staphylococcus aureus* Δ*hemB* small colony variant

2003 Gordon Conference, Oxford, UK
Poster presentation: *In vitro* assembly of the complete pentaglycine interpeptide bridge containing cell wall precursor of *Staphylococcus aureus*

2003 SGM, Basel, Switzerland
Poster presentation: The transcriptional pattern in a *Staphylococcus aureus* SCV corresponds to early growth phase

Stipends

Competitive post-doctoral stipends of the “Theodor und Ida Herzog-Egli” as well as the “EMDO” foundations, Zürich.

9.9. Tables and Figures

9.10. Publications

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The σ^B Regulon Influences Internalization of *Staphylococcus aureus* by Osteoblasts

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Individual strains of *Staphylococcus aureus* have different capacities to become internalized by osteoblasts. Here we report that the levels of σ^B expressed by *S. aureus* correlate with the capacity of this bacterium to be internalized by osteoblasts. However, σ^B is not essential for internalization and does not necessarily account for the differences in the capacities of strains to be internalized.

Staphylococcus aureus is an important human pathogen that causes a range of infections, including those of bone. *S. aureus* bone infections can be extremely difficult to treat, requiring prolonged antibiotic treatment and surgical intervention (18). We, and others, have shown previously that *S. aureus* is internalized by the bone-forming cells, osteoblasts (10, 14, 15, 19). The capacity of *S. aureus* to be internalized by osteoblasts has been proposed as an explanation for the recurrent nature of, and the difficulty in treating, bone infections with this organism.

We have previously shown that osteoblasts employ a receptor-mediated pathway in the uptake of *S. aureus* (15) and that the expression of fibronectin binding proteins (FnBPs) by the bacterium is essential to the process of internalization (1). However, the capacities of different isolates of *S. aureus* to be internalized by osteoblasts varied and could not be accounted for by differences in the levels of expression of FnBPs by these strains. In fact, *S. aureus* strain LS-1, which expressed lower levels of FnBPs, was internalized to a greater extent than strain 8325-4 (1). Although the mechanism underlying the differences in the capacities of these two strains to be internalized is unknown, it is interesting that strain 8235-4 is a derivative of NCTC8325, which is a natural *rsbU* mutant (13, 17).

In *Bacillus subtilis*, a network of protein-protein interactions regulates the activity of the alternative sigma factor, σ^B, post-translationally. One of the proteins in this network, RsbU, is essential for the activation of σ^B. It has recently been shown that σ^B activity in *S. aureus* also depends on RsbU (13). Derivatives of *S. aureus* NCTC8325 have an 11-bp deletion in *rsbU*, resulting in the introduction of a stop codon into this gene, and hence these strains are essentially deficient in σ^B activity.

In many bacteria the alternative sigma factors of RNA polymerase are important in cell adaptation to environmental stress (20). Association of σ^B with the core RNA polymerase results in the recognition of a specific subset of promoters and to the initiation of transcription of their genes. To date only one alternative sigma factor has been identified in *S. aureus*, σ^B. Although σ^B has primarily been associated with responses to environmental stress, it has been shown that expression of the global virulence regulator SarA is influenced by σ^B in *S. aureus* (8, 9, 12, 17), and it has been suggested that σ^B functions as a global regulator of virulence genes in this bacterium (16). It has recently been demonstrated that σ^B expression influences transcription of both the *sar* and *agr* loci, and it has been suggested that σ^B might prolong the production of cell surface proteins such as the FnBPs, while preventing upregulation of secreted exoproteins (4).

Given the apparent importance of σ^B in the regulation of virulence genes in *S. aureus*, we have examined the hypothesis that σ^B activity may influence the capacity of *S. aureus* to be internalized by osteoblasts and thus account for our previous observation of strain-dependent differences in this capacity (1).

The bacterial strains used in this study are listed in Table 1. Strain BB1591 was obtained by phage 80α-mediated transduction of (Δ*rsbUVWsigB*)::*ermB* from IK181 (16) into strain LS-1, selecting for erythromycin resistance. Strain MB258 was obtained by transduction of the reporter construct *asp23p*::pBT*asp23p-luc*$^+$ from MB61 (13) into strain LS-1, selecting for tetracycline resistance. The levels of σ^B activity in *S. aureus* strains were analyzed during growth by using an *asp23* reporter gene system as previously described (13). *S. aureus* strain BB255 is our laboratory stock of NCTC8325 and carries a mutation in *rsbU*5. Table 2 shows that the MB33 reporter strain of BB255 expressed very low levels of σ^B activity. Complementation of this strain with an intact *rsbU* allele from strain COL, as in strain GP268, resulted in expression of significant levels of σ^B activity, as indicated by the reporter strain MB49 (Table 2). The level of σ^B activity in MB49 is similar to that found in other RsbU$^+$ strains of *S. aureus* (13). Replacement of the σ^B operon in *S. aureus* strain BB255 with an erythromycin cassette, as in strain IK181, resulted in no expression of σ^B activity as shown by the reporter strain MB90 (Table 2). *S. aureus* strain MB138, BB255 with a point mutation in the anti-sigma factor *rsbW*, produced high levels of σ^B activity (Table 2). *S. aureus* strain LS-1, represented by strain MB258, produced levels of σ^B activity which were similar to those of strain GP268, as indicated by the reporter strain

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TABLE 1. Strains used in this study

Strain	Relevant genotype and phenotype[a]	Reference(s) or source
BB255	Laboratory stock of NCTC8325, *rsbU*	2
MB33	BB255 *asp23*$^+$ *asp23p*::pE*Casp23p-luc*$^+$ Em^r	13
IK181	BB255 (Δ*rsbUVWsigB*)::*erm*(B) Em^r	16
MB61	RN4220 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r	13
MB90	IK181 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r Em^r	13
GP268	BB255 (*rsbU*$^+$*V*$^+$*W*$^+$*sigB*$^+$)-*tetL* Tc^r	13
MB49	GP268 *asp23*$^+$ *asp23*$^+$ *asp23p*::pEC*asp23p-luc*$^+$ Em^r Tc^r	13
MB138	MB33 *rsbW7* Em^r	3
LS-1		6, 7
MB258	LS-1-*asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r	This study
BB1591	LS-1 (Δ*rsbUVWsigB*)::*erm*(B) Em^r	This study
MB259	BB1591 *asp23*$^+$ *asp23p*::pBT*asp23p-luc*$^+$ Tc^r Em^r	This study

[a] *asp23* is the gene for the 23-kDa alkaline shock protein of *S. aureus.*

MB49 (Table 2). Replacement of the σ^B operon in *S. aureus* LS-1 with an erythromycin cassette, as in strain BB1591, led to a complete loss of σ^B activity as indicated by the reporter strain MB259 (Table 2).

The capacity of *S. aureus* strains to be internalized by the osteoblastic cell line MG63 was determined as previously described (1) with the modification that overnight cultures of bacteria were diluted 1:20 in 10 ml of fresh brain heart infusion and grown to an optical density at 600 nm of approximately 1 before use. *S. aureus* strain BB255 was internalized by osteoblasts as shown in Fig. 1. Strain GP268 (BB255 *rsbU*$^+$) had a twofold-greater capacity to be internalized by osteoblasts than did BB255 (Fig. 1). The capacity of strain IK181 (BB255 Δ*rsbUVWsigB*) to be internalized by osteoblasts was slightly lower than that of strain BB255, although the difference was not statistically significant (Fig. 1). *S. aureus* strain MB138 (BB255 *rsbW*) had a 10-fold-greater capacity to become internalized by osteoblasts than did strain BB255 (Fig. 1). To determine if σ^B activity had an effect on the ability of *S. aureus* to grow and/or survive within osteoblasts, cocultures were extended for a period of up to 6 h as previously described (1). There was no significant difference in the percentages of the natural *rsbU* mutant BB255 and its isogenic mutants GP268 and MB138 recovered, at hourly intervals up to 6 h, from the number recovered after 2 h (data not shown).

It has previously been shown that *S. aureus* strain LS-1 has a

TABLE 2. Strain-dependent differences in σ^B activity

Strain	Relevant genotype[a]	σ^B activity[b]
MB33	BB255	34.0 ± 3.00
MB49	BB255 *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	301.3 ± 23.8
MB90	BB255 Δ*rsbUVWsigB*	0.62 ± 0.10
MB138	BB255 *rsbW7*	1,084.0 ± 46.5
MB258	LS1 *rsbU*$^+$*V*$^+$*W*$^+$ *sigB*$^+$	256.6 ± 17.2
MB259	LS1 Δ*rsbUVWsigB*	0.31 ± 0.14

[a] Detailed relevant genotypes and phenotypes are listed in Table 1.

[b] σ^B transcriptional activity (relative light units) was determined from cells grown to an optical density at 600 nm of 1.5 by measuring the luciferase activity of Luc$^+$, the product of the *luc*$^+$ reporter gene fused to the σ^B-dependent promoters of *asp23.* The values shown are the results of four independent assays.

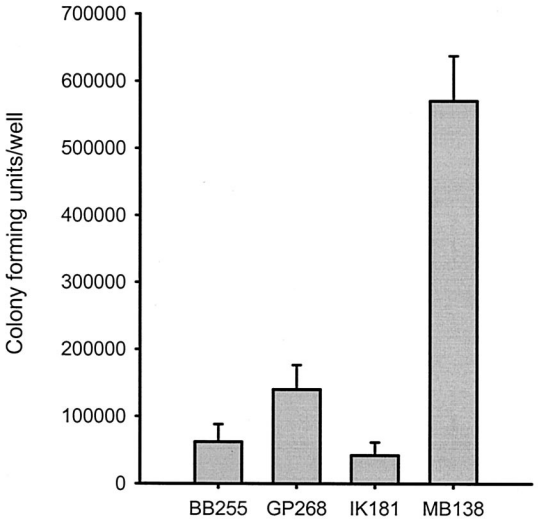

FIG. 1. Capacity of *S. aureus* strain BB255 and its isogenic mutants to be internalized by osteoblasts. The isogenic strains BB255 (*rsbU*), GP268 (BB255 *rsbU*$^+$), IK181 (BB255 Δ*rsbUVWsigB*), and MB138 (BB255 *rsbW*) were cocultured with osteoblasts at a multiplicity of infection of 100:1. The figure shows the results from one representative experiment of at least three, and the data are the means and standard deviations of three replicate cultures.

10-fold-greater capacity to be internalized by osteoblasts than does the NCTC8325 derivative 8325-4 (1). In this study we compared the capacity of the NCTC8325 derivative BB255 to be internalized by osteoblasts with that of LS-1. Figure 2 shows that *S. aureus* strain LS-1 had a greater capacity to be internalized than did strain BB255 (sevenfold) or its *rsbU*$^+$ derivative GP268. Strain BB1591, LS-1 in which the σ^B operon had

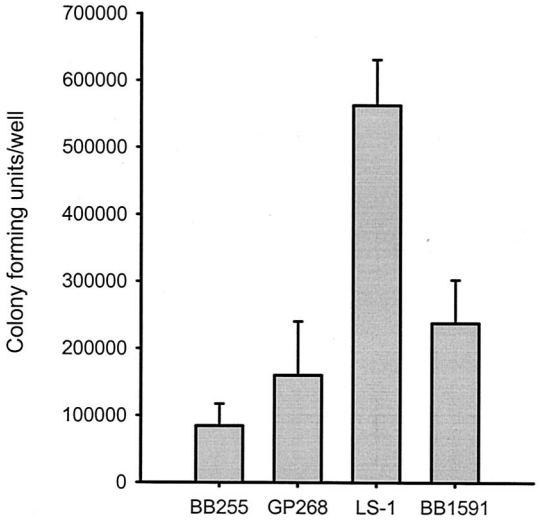

FIG. 2. Comparison of the capacities of *S. aureus* strains BB255 and LS-1 and their respective isogenic mutants to be internalized by osteoblasts. *S. aureus* strains BB255 (*rsbU*), GP268 (BB255 *rsbUVWsigB*$^+$), LS-1, and BB1591 (LS-1 Δ*rsbUVWsigB*) were cocultured with osteoblasts at a multiplicity of infection of 100:1. The figure shows the results from one representative experiment of at least three, and the data are the means and standard deviations of three replicate cultures.

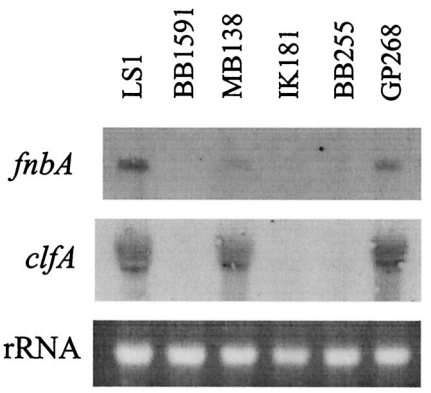

FIG. 3. Northern blot analyses of *fnbA* and *clfA* expression by *S. aureus* strains LS1, BB1591 (LS1 Δ*rsbUVWsigB*), MB138 (BB255 *rsbW*), IK181 (BB255 Δ*rsbUVWsigB*), BB255 (*rsbU*), and GP268 (BB255 *rsbU*$^+$).

been deleted, had a twofold-lower capacity to be internalized by osteoblasts than did its parent LS-1 (Fig. 2).

One mechanism by which the σ^B operon may influence internalization of *S. aureus* by osteoblasts is alteration of the expression of bacterial cell surface proteins such as the FnBPs. To investigate this possibility, Northern blot analysis for *fnbA* and *clfA* expression was performed. Bacterial overnight cultures were diluted to an optical density at 600 nm of 0.1 and grown at 37°C. RNA samples were prepared from cells collected after 1 h and after 8 h. Specific, digoxigenin-labeled *fnbA* and *clfA* probes were used for hybridizing 1-h and 8-h RNA extracts (7 μg), respectively. As can be seen in Fig. 3, the levels of expression of *fnbA* and *clfA* were highest in strains LS-1, MB138, GP268, and MB79, all of which possessed high σ^B activity. The capacities of *S. aureus* strains to bind to fibronectin were determined as previously described (1). The capacity of the *rsbU* mutant BB1591 to bind fibronectin was reduced to 24% ± 6% (mean ± standard deviation) of that of the wild-type *rsbU*$^+$ strain LS-1. Similarly the capacity of the natural *rsbU* mutant BB255 to bind to fibronectin was only 35% ± 9% (mean ± standard deviation) of that of the *rsbU*-complemented strain GP268.

It has been postulated that the capacity of *S. aureus* to be internalized by mammalian cells may contribute to the pathogenicity of this organism. In particular this capacity could account for the persistent nature of some *S. aureus* infections. The molecular details of the bacterial-host cell interactions that result in uptake of *S. aureus* by host cells are beginning to be elucidated. It has become evident that the FnBPs of *S. aureus* play an important, if not essential, role in the uptake process. However, we have previously reported that strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts do not necessarily correlate with the levels of expression of the FnBPs, suggesting that other factors are also important in this process (1). Since we were aware that one of the strains used in the previous study was an *rsbU* mutant that was deficient in σ^B expression, we have examined the possibility that this may account for strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts.

Complementation of the natural *rsbU* mutant BB255 with a functional *rsbU* gene resulted in the expression of significant levels of σ^B activity and increased the capacity of this strain to be internalized by osteoblasts by twofold. Deletion of the entire σ^B operon in BB255 resulted in no expression of σ^B activity and had little effect on the capacity of this strain to be internalized by osteoblasts. On the other hand, an isogenic strain with a point mutation in *rsbW*, which resulted in the expression of very high levels of σ^B activity, had a significantly increased capacity (5- to 10-fold) to be internalized by osteoblasts compared to that of the parental strain. Upon entry into the osteoblast *S. aureus* is found within intracellular vacuoles, as well as free within the cytosol. If the intracellular vacuoles containing *S. aureus* fuse with the lysosomal system, then the bacteria may be subject to environmental stresses such as low pH. Since the alternative sigma factor, σ^B, regulates the expression of general stress response genes (11, 12), it might affect the ability of *S. aureus* to survive and/or grow within the intracellular environment of the osteoblast. If this were the case, the numbers of staphylococci recovered from within osteoblasts incubated with isogenic strains producing high levels of σ^B activity would not be a reflection of the capacity of these bacteria to be internalized by osteoblasts. However, our studies show that the levels of σ^B expressed by isogenic mutants do not affect the ability of *S. aureus* to survive and/or grow within the intracellular environment of the osteoblasts. These data taken together clearly demonstrate that the level of σ^B activity expressed by *S. aureus* affects the capacity of this bacterium to be internalized by osteoblasts.

Given that the level of expression of σ^B activity in *S. aureus* affects the capacity of this bacterium to be internalized by osteoblasts, it could account for strain-dependent differences in this capacity. Our previous studies and data presented herein show that *S. aureus* strain LS-1 has a much higher capacity (7- to 10-fold) to be internalized by osteoblasts than do the NCTC8325 derivatives 8325-4 (1) and BB255. *S. aureus* strain LS-1 also produces significant amounts of σ^B activity in comparison to the NCTC8325 derivatives. In fact the levels of σ^B produced by LS-1 are equivalent to those produced by BB255 complemented with an intact *rsbU* gene as in GP268. However LS-1 has a three- to fourfold-higher capacity to be internalized by osteoblasts than does strain GP268. Deletion of the σ^B operon in LS-1 resulted in a complete loss of σ^B activity but only halved its capacity to be internalized by osteoblasts. These data demonstrate that, while σ^B activity affects the capacity of *S. aureus* to be internalized by osteoblasts, it cannot fully account for the strain-dependent differences that have previously been reported (1). Thus, other as-yet-undefined factors must also play a role in the strain-dependent differences in the capacity of *S. aureus* to be internalized by osteoblasts. In the present study we have also shown that the σ^B operon affects the level of expression of two microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), *fnbA* and *clfA*. Furthermore we have found that the capacities of *S. aureus* strains to bind to fibronectin correlate with their σ^B activities. We have previously reported that at least two MSCRAMMs, the FnBPs A and B, play an important role in the process of internalization of *S. aureus* by osteoblasts. This suggests that one possible mechanism by which the σ^B regulon may influence the internalization of *S. aureus* by osteoblasts is through alteration in the level of expression of MSCRAMMs.

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In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly_5) of *Staphylococcus aureus*

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Summary

Staphylococcus aureus **peptidoglycan is cross-linked via a characteristic pentaglycine interpeptide bridge. Genetic analysis had identified three peptidyltransferases, FemA, FemB and FemX, to catalyse the formation of the interpeptide bridge, using glycyl t-RNA as Gly donor. To analyse the pentaglycine bridge formation *in vitro*, we purified the potential substrates for FemA, FemB and FemX, UDP-MurNAc-pentapeptide, lipid I and lipid II and the staphylococcal t-RNA pool, as well as His-tagged Gly-tRNA-synthetase and His-tagged FemA, FemB and FemX. We found that FemX used lipid II exclusively as acceptor for the first Gly residue. Addition of Gly 2,3 and of Gly 4,5 was catalysed by FemA and FemB, respectively, and both enzymes were specific for lipid II-Gly_1 and lipid II-Gly_3 as acceptors. None of the FemABX enzymes required the presence of one or two of the other Fem proteins for activity; rather, bridge formation was delayed in the *in vitro* system when all three enzymes were present. The *in vitro* assembly system described here will enable detailed analysis of late, membrane-associated steps of *S. aureus* peptidoglycan biosynthesis.**

Introduction

The *Staphylococcus aureus* peptidoglycan comprises about 50% of the entire cell wall and forms a multilayered, highly cross-linked sacculus, which protects the bacterium from osmotic pressure and determines its shape. It consists of 20 or more layers of linear glycan chains with alternating units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). The carboxylic group of *N*-acetylmuramic acid is substituted with the stem pentapeptide L-Ala-D-iGlu-L-Lys-D-Ala-D-Ala. A pentaglycine interpeptide, branching off the ε-amino group of the L-Lys of the stem peptide, connects one peptidoglycan chain to the D-Ala in position 4 of a neighbouring chain (Fig. 1).

The flexible interpeptide allows a high, three-dimensional cross-linking of the peptidoglycan, characteristic of the staphylococcal cell wall. It also acts as attachment site for various cell wall-sorted proteins, which are important in infection and virulence of this pathogen (Perry *et al*., 2002; Roche *et al*., 2003). Moreover, the interpeptide is essential for the full expression of methicillin resistance in methicillin-resistant *S. aureus* (MRSA). Shortening of the pentaglycine chain impairs growth, leading to aberrant septum formation and lowered methicillin resistance levels (Maidhof *et al*., 1991). The interpeptide is essential for *S. aureus*; survival with a shortened bridge reduced to only one glycine requires as yet uncharacterized compensatory mutations (Ling and Berger-Bächi, 1998).

Extensive genetic analysis and characterization of mutant cell walls suggested that the pentaglycine interpeptide is synthesized in a sequential fashion by proteins belonging to the new FemABX family of non-ribosomal peptidyltransferases (Hegde and Shrader, 2001; Rohrer and Berger-Bächi, 2003a). FemX, encoded by the essential gene *fmhB*, was found to be essential for incorporation of the first glycine to the stem peptide (Rohrer *et al*., 1999). The second factor, FemA, was proposed to catalyse the addition of the second and third glycine (Maidhof *et al*., 1991; Stranden *et al*., 1997), whereas FemB is supposed to add glycines four and five (Fig. 1) (Henze *et al*., 1993). The peptidyltransferases use glycyl-tRNA as donor in a ribosome-independent reaction (Kamiryo and Matsuhashi, 1972).

Biochemical analysis of the staphylococcal FemABX system has so far been hampered by the fact that the presumed substrates for glycine addition, the lipid-bound cell wall precursors lipid I and lipid II, could not be made available.

Homologues of FemA, FemB and FemX have been identified in all Gram-positive bacteria, which produce a

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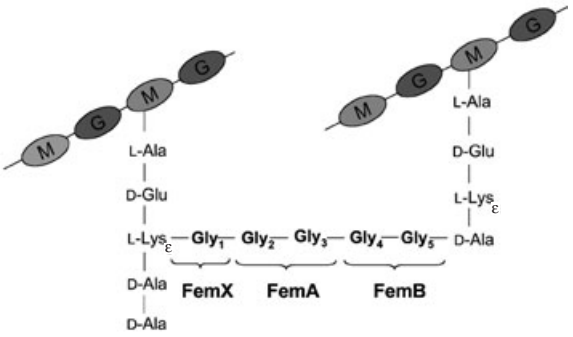

Fig. 1. Structure of *S. aureus* and *S. simulans* 22 interpeptide bridge.

branched-chain peptidoglycan with either glycine and/or L-amino acids in the interpeptide (Rohrer and Berger-Bächi, 2003b). Interestingly, the pneumococcal MurMN, FibAB respectively (Filipe *et al*., 2000; Weber *et al*., 2000), were found to be associated in that species with penicillin resistance as well. Staphylococcal strains producing glycyl-glycine endopeptidases, such as lysostaphin (Thumm and Götz, 1997) or ALE1 (Sugai *et al*., 1997), protect their cell walls by FemABX-like immunity factors, termed Lif or Epr, which insert serine in place of glycine at positions 3 and 5 in the interpeptide (Ehlert *et al*., 2000). Lif was shown to be able to complement a *femB* mutant in *S. aureus* (Tschierske *et al*., 1997).

Pentapeptide modification occurs during cell wall biosynthesis at the inner face of the cytoplasmic membrane before the monomeric peptidoglycan unit is translocated across the membrane and assembled into the growing peptidoglycan network. The soluble FemX of *Lactobacillus viridescens* (Hegde and Shrader, 2001) and the *Enterococcus faecalis* BppA1 and BppA2 (Bouhss *et al*., 2001) have been shown to use UDP-MurNAc-pentapeptide as substrate for the synthesis of branched peptidoglycan precursors. The inability to add the first glycine to this soluble substrate by the staphylococcal FemX (Hegde and Shrader, 2001) suggests that FemABX peptidyltransferases of *S. aureus* may, in contrast to the previous systems, be membrane associated, and the acceptor molecule may be one of the lipid-bound cell wall precursors, lipid I or lipid II. Lipid I (undecaprenylphosphate-MurNAc-pentapeptide) is formed in the first lipid-linked step of cell wall synthesis by MraY, which transfers the soluble UDP-MurNAc-pentapeptide to the lipid carrier undecaprenylphosphate (C55-P). The translocase MurG subsequently links UDP-activated *N*-acetyl-glucosamine (UDP-GlucNAc) to the muramoyl moiety of lipid I, thus yielding lipid II (undecaprenylphosphate-GlucNAc-MurNAc-pentapeptide) (van Heijenoort and Gutmann, 2000).

In order to analyse the individual steps of interpeptide formation in *S. aureus* and to identify the correct substrates, we purified lipid I and lipid II, the essential enzymes FemA, FemB, FemX, the glycyl-tRNA synthetase and completely reconstituted *in vitro* the membrane-bound steps of staphylococcal cell wall synthesis. We found the Fem peptidyltransferases to be active individually and highly substrate specific and identified lipid II as the only substrate of the *S. aureus* peptidyltransferases.

Results

Preparation of lipid I and lipid II – setup of the assays and detection system

Membrane preparations possess enzymatic activity (MraY and MurG) for the formation of cell wall precursors (van Heijenoort *et al*., 1978), and membranes of *S. aureus* have been reported to contain Fem protein activities and to synthesize glycine-labelled lipid intermediates when complemented with soluble cellular fraction (Matsuhashi *et al*., 1967). As shown with *Staphylococcus simulans* 22 membranes (Fig. 2), the lipid carrier molecule (C55-P) remained unmodified in the absence of UDP-activated precursors. Upon addition of UDP-MurNAc-PP, C55-P was transferred into lipid I; the conversion was incomplete because of the reversibility of the MraY-catalysed reaction (Pless and Neuhaus, 1973). When UDP-GlcNAc was also

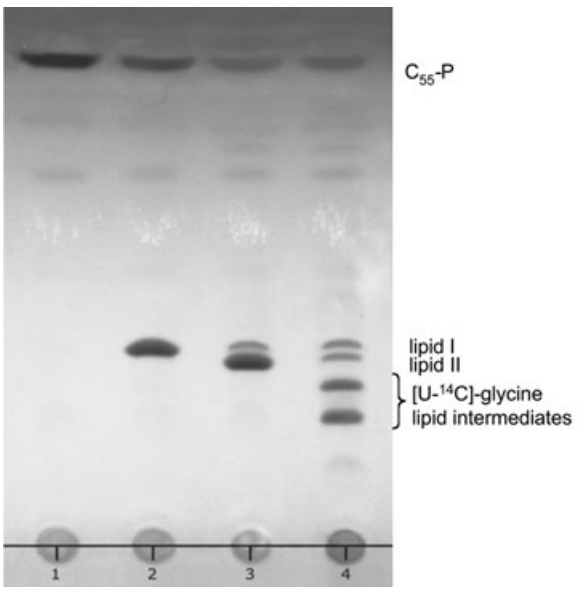

Fig. 2. TLC of [U-^{14}C]-glycine-labelled lipid intermediates. Undecaprenylphosphate (10 nmol; C55-P) was incubated *in vitro* with membranes (360 μg of protein) of *S. simulans* 22 for 60 min in the presence of 50 nmol of glycine lacking the UDP-activated precursors (lane 1). Lipid I was synthesized upon addition of UDP-MurNAc-PP (lane 2) and lipid II by addition of UDP-MurNAc-PP and UDP-GlcNAc (lane 3). Two glycine-labelled lipid intermediates migrating more slowly than lipid I and lipid II were separated after incubation with UDP-MurNAc-PP and UDP-GlcNAc and addition of supernatant I (330 μg of protein) (lane 4).

present, most of the lipid I was converted to lipid II, which migrates somewhat more slowly. Using lipid II from a preparative batch, its position on the chromatogram was verified, and its molecular mass was confirmed by mass spectroscopy (Fig. 3.1).

When radiolabelled glycine was added to the assay, no further bands were observed (Fig. 2), and both bands representing lipid I and lipid II did not contain radiolabel. In contrast, when the assay mixture was supplemented with the soluble cytosolic fraction obtained after low-speed centrifugation (supernatant I), two additional bands occurred with Rf-values lower than that of lipid II (Fig. 2, lane 4). Both bands were radiolabelled, strongly suggesting that they represent lipid I/lipid II intermediates with different segments of the glycine interpeptide bridge attached. Moreover, from the intensities of the various bands obtained after phosphomolybdic acid (PMA) staining, it appeared that lipid II was the major substrate for the Fem-catalysed reaction, as it was strongly reduced on the chromatogram in favour of the two newly formed glycine-containing intermediates.

In vitro *activity of FemX, FemA and FemB*

Although the above system, based on the use of soluble cytosolic fractions, was sufficient to determine that all factors necessary for interpeptide formation (i.e. the Fem factors, glycyl-tRNA-synthetase and tRNA) were present,

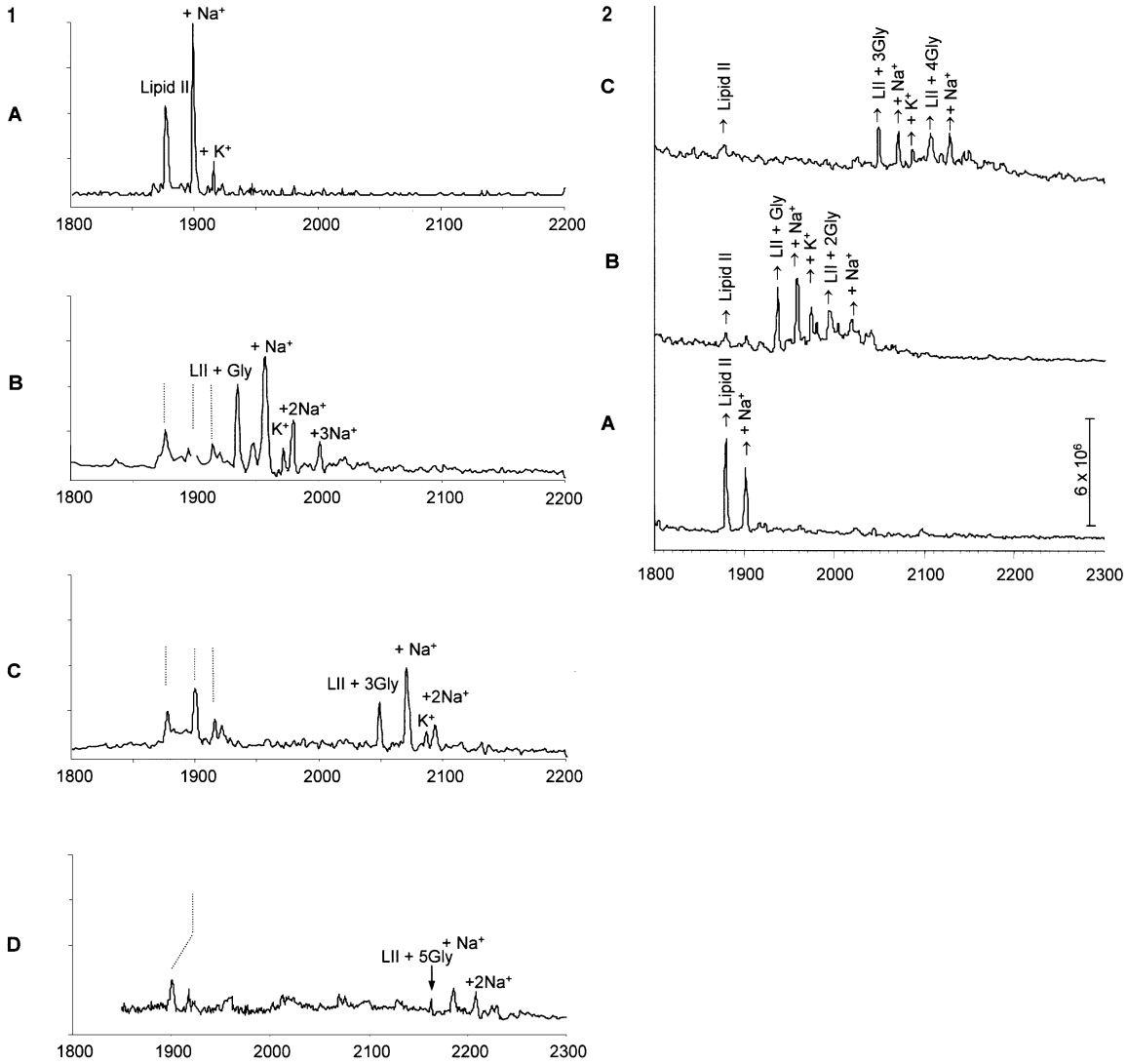

Fig. 3. 1. ES-MS spectra were obtained with an API-I single quadrupole instrument (PE-SCIEX) running in a positive mode. Peaks at *m/z* 1878 correspond to lipid II (A), at *m/z* 1935 to lipid II-Gly_1 (B), at *m/z* 2049 to lipid II-Gly_3 (C) and at *m/z* 2163 to lipid II-Gly_5 (D). Other peaks correspond to the non-covalent sodium or potassium ion adducts.
2. With prolonged incubation time (90 min) and elevated enzyme concentrations (molar ratio of enzyme:lipid II, 1:50), lipid II-Gly_2 (B) at *m/z* 1992 and lipid II-Gly_4 (C) at *m/z* 2106 were detected in addition to the correct products.

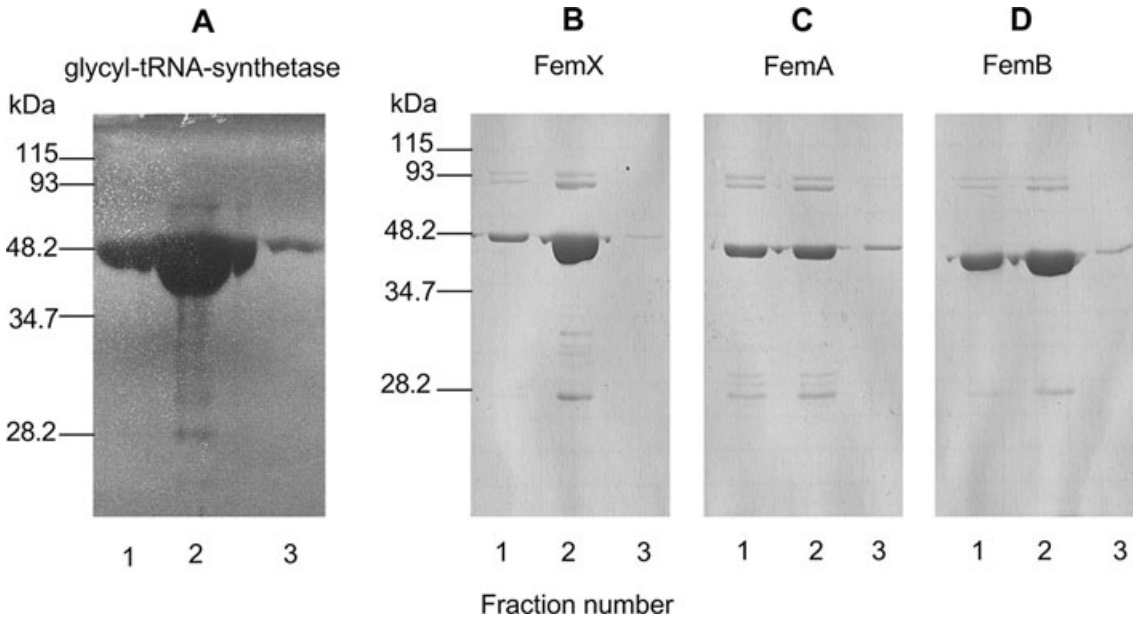

Fig. 4. **10**% SDS–PAGE of recombinant proteins. His-tagged enzymes were purified on a Ni-NTA column as described in *Experimental procedures*. Three 500 μl fractions were collected, to which an equal amount of 100% glycerol was added for preservation at –20°C. Gly-tRNA-synthetase fractions (10 μl) and 1 μl of FemABX fractions were loaded on to the gel. Prestained broad-range standard marker from Bio-Rad was used to estimate molecular masses. Fractions 1–3 of Gly-tRNA-synthetase (A). Fractions 1–3 of FemX (B). Fractions 1–3 of FemA (C). Fractions 1–3 of FemB (D). The following fractions were selected for further experiments: A2 (Gly-tRNA-synthetase); B2 (FemX); C1 and C2 (FemA); D1 and D2 (FemB).

it did not allow for a detailed step-by-step analysis of bridge formation. In particular, the presence of MurG activity in the supernatant prevented unequivocal determination of whether lipid I or lipid II was the substrate for FemX. High-speed ultracentrifugation (supernatant II) clearly reduced MurG activity; however, the overall reaction was strongly reduced, presumably because of simultaneously removing the Fem enzymes and/or glycyl-tRNA-synthetase and tRNAs (data not shown).

We therefore His-tagged and purified FemX, FemA, FemB and the glycyl-tRNA-synthetase of *S. aureus* NCTC 8325 (Fig. 4). In addition, total tRNAs were isolated from both *S. simulans* 22 and *S. aureus*. Replacing the supernatant by recombinant FemX, FemA and FemB, glycyl-tRNA-synthetase and purified tRNAs resulted in the formation of glycine-labelled lipid intermediates (Fig. 5). Under the conditions chosen, i.e. with an excess of [^{14}C]-glycine and a defined amount of 5 nmol of lipid II in the system, the availability of dedicated glycyl-tRNA was rate limiting. At a concentration of 25 μg of total tRNA per assay volume, the maximum amount of pentaglycine bridge formation was obtained. There was no difference observed using purified tRNAs from *S. simulans* 22 or *S. aureus*. Assuming that all lipid II molecules were available,

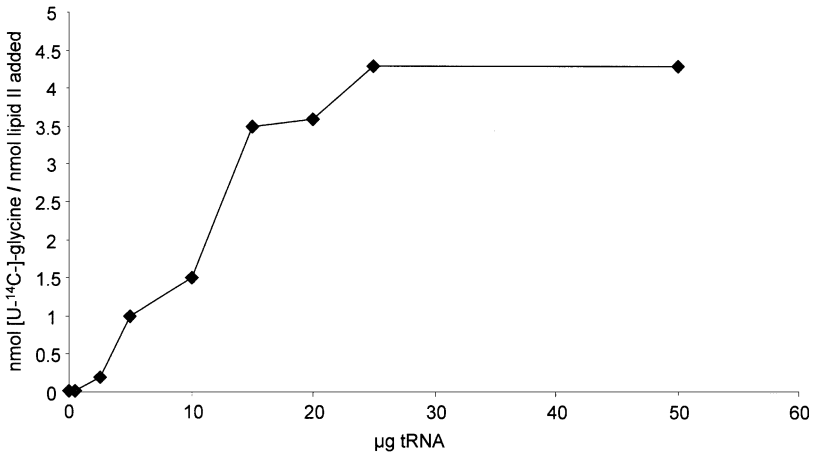

Fig. 5. Influence of increasing tRNA concentrations on the FemXAB reaction. Purified lipid II was incubated with [U-^{14}C]-glycine in the presence of FemXAB and recombinant tRNA synthetase with increasing concentrations of purified tRNAs. Reaction products were separated by TLC, and incorporation of [U-^{14}C]-glycine was determined by scintillation counting.

a 5:1 ratio of glycine to lipid II was expected in the final product. As indicated in Fig. 5, the maximum amount of glycine incorporation obtained in the *in vitro* system corresponded to an average of 4.3 mol of glycine incorporated per mol of lipid II.

The cell wall precursor lipid II is the only substrate of FemX

Identification of the substrates of the FemX- and subsequently of the FemA- and FemB-catalysed reactions was achieved by incubation of lipid I or lipid II (Fig. 6) in the presence of glycyl-tRNA-synthetase and tRNAs, with FemX, FemX and FemA and FemX, FemA and FemB. Analysis of the reaction products by thin-layer chromatography (TLC) after incubation of lipid I with the Fem proteins identified lipid I as a poor substrate for FemX, and no further incorporation of glycine was detected after incubation with additional FemA and FemB (Fig. 6B).

In contrast, incubation of lipid II resulted in the formation of glycine lipid II intermediates migrating stepwise more slowly in the TLC system upon addition of glycine to the pentapeptide side-chain of lipid II (Fig. 6A). The molar ratio of radiolabelled glycine to lipid intermediate indicated that FemX (glycine:lipid II, 1.3:1) added the first glycine to lipid II. The glycine:lipid incorporation ratios for the FemXA and the FemABX reactions were 2.76:1 and 4.17:1 as expected after the formation of lipid II-Gly_3 and -Gly_5 (Fig. 6B). Lipid II itself, i.e. without the first glycine added by FemX, was not used by FemA and Fem B, either on their own or combined. We only observed background Gly incorporation levels in the range of 0.02–0.2 Gly incorporated per lipid II molecule. Although the incorporation levels were somehow lower than expected theoretically, especially for the FemABX reaction, analysis of the products by mass spectrometry confirmed the formation of lipid II-Gly_1 by FemX, lipid II-Gly_3 by FemXA and lipid II-Gly_5 by FemXAB (Fig. 3.1). It should be noted that determination of glycine incorporation ratios required, first, extraction of radiolabelled intermediates from the synthesis assay using n-butanol/pyridine-acetate, subsequent TLC and, finally, counting of excised bands (see *Experimental procedures*); this procedure is likely to cause some loss of radiolabelled products and, obviously, the extraction yields decrease with increasing bridge length of the products.

Lipid II with intermediate glycine contents (lipid II-Gly_2 and lipid II-Gly_4) was not detected under these conditions (Fig. 3.1). However, when the assay conditions were changed (90 min incubation and a lipid II:enzymes ratio of 50:1), such products were detectable (Fig. 3.2).

Obviously, under the experimental conditions chosen, i.e. lipid II in micellar form and in the presence of 0.8% Triton X-100, FemX not only accepts lipid II as a substrate but also lipid II-Gly_1. Accordingly, after 90 min of incubation, the reaction products of FemXA contained lipid II-

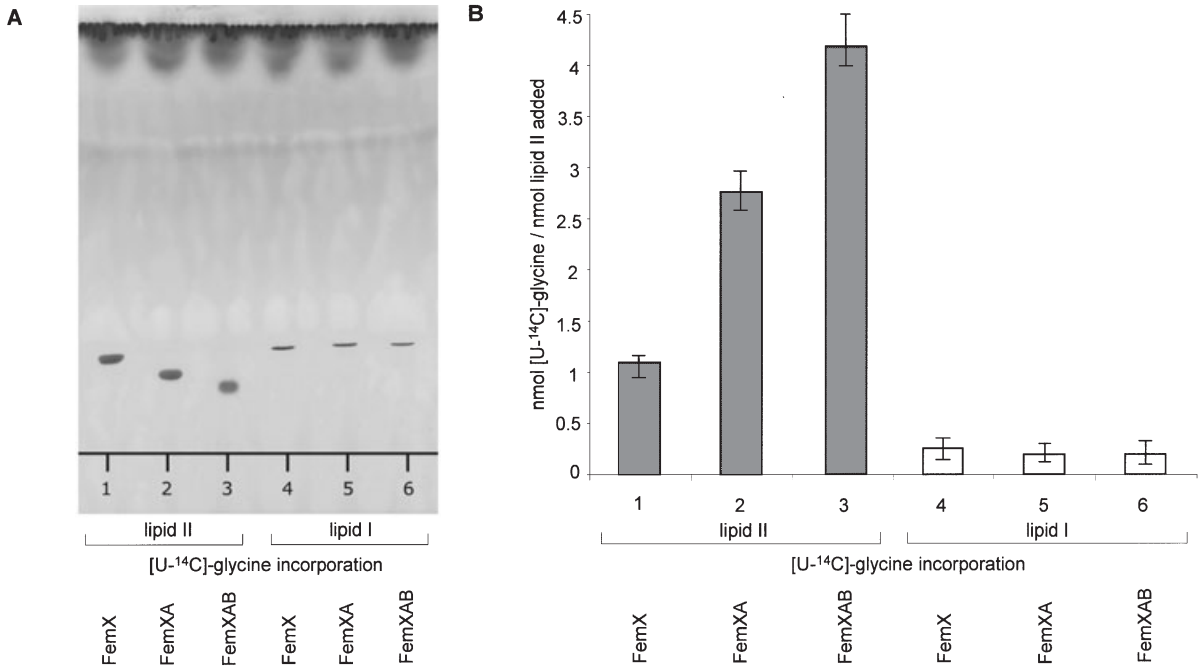

Fig. 6. A. TLC of glycine-labelled lipid intermediates synthesized by recombinant FemX, FemA and FemB. Purified lipid II (lanes 1–3) and lipid I (lanes 4–6) were incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA-synthetase and purified tRNA with FemX (lanes 1 and 4), FemX and FemA (lanes 2 and lane 5) and FemX, FemA and FemB (lanes 3 and 6).
B. [U-^{14}C]-glycine:lipid ratio of the analysed glycine lipid intermediates. Mean values of five experiments are given.

Gly_3 and lipid II-Gly_4 (Fig. 3.2). Obviously, FemA can also accept lipid II-Gly_2 as a substrate and then incorporate two additional glycine residues.

As reported previously, UDP-MurNAc-PP is the substrate for the soluble recombinant FemX proteins of *Lactobacillus viridescens* and *Enterococcus faecalis* (Hegde and Shrader, 2001; Bouhss *et al.*, 2002). To exclude UDP-MurNAc-PP as a substrate for the Fem proteins of *S. aureus*, 5 nmol of lipid II was incubated with FemXAB, glycyl-tRNA-synthetase and tRNAs in the presence of 5–100 nmol of purified UDP-MurNAc-PP isolated from *S. simulans* 22. Analysis of the reaction products revealed that the addition of glycine to lipid II was not affected even in the presence of a 20-fold molar excess of UDP-MurNAc-PP. This competition experiment demonstrates that the FemXAB enzymes of *S. aureus* catalyse the addition of glycine to the pentapeptide side-chain only after the formation of lipid II. This result is in good agreement with the fact that UDP-MurNAc hexapeptides were detectable in the cytoplasm of *L. viridescens* (Plapp and Strominger, 1970), but not in *S. aureus* (Stickgold and Neuhaus, 1967) or in the staphylococcal strains used in this study (data not shown).

Time course of the FemXAB reactions

The formation of the Gly-containing lipid II intermediates was followed over time (Fig. 7). Initial Gly addition by FemX was already completed after 5 min and resulted in a glycine:lipid II ratio of 1.22:1(see above). In contrast, the presence of FemA in the synthesis assay decreased the formation of lipid II-Gly_1 reaction products, indicating that, in our *in vitro* system, the presence of FemA interferes with Gly addition by FemX. The maximum level of the FemXA reaction was reached after an incubation period of 60 min with a final glycine:lipid II ratio of 3:1. An even stronger effect was observed upon addition of FemB. The FemX reaction was retarded further, and glycine lipid II intermediates were not detected before 15 min of incubation. The synthesis was not completed within 90 min of incubation.

Analysis of the individual Fem protein reactions

As the maximum catalytic activity of FemX was observed in the absence of FemA and FemB (Fig. 7), we purified the unlabelled FemX and FemA reaction products to study the FemA and FemB reactions independently (Fig. 8). Using the standard assay, comparable amounts of labelled Gly per lipid were added to lipid II-Gly_1 by FemA alone (1.73:1) and by FemA when FemX was present (1.90:1). Again, the observed incorporation was somewhat lower than the theoretically expected value of two Gly per lipid II-Gly_1 incorporated, as discussed above. This result clearly demonstrates that FemA activity *in vitro* does not depend on the presence of FemX. Neither FemX nor FemB catalysed further addition of glycine to lipid II-Gly_1, proving the preference of FemX for lipid II and of FemB for lipid II intermediates containing at least three Gly.

The overall incorporated radioactivity by FemB and FemAB suggested that only one more Gly residue may have been added to the lipid II-Gly_3 (Fig. 8); however, mass spectroscopy clearly showed that only lipid II-Gly_5 occurred as a product (Fig. 3.1). This further indicates that extraction yields from the synthesis assay decrease strongly with interpeptide chain length (see also above).

Discussion

The FemABX peptidyltransferases are interesting drug

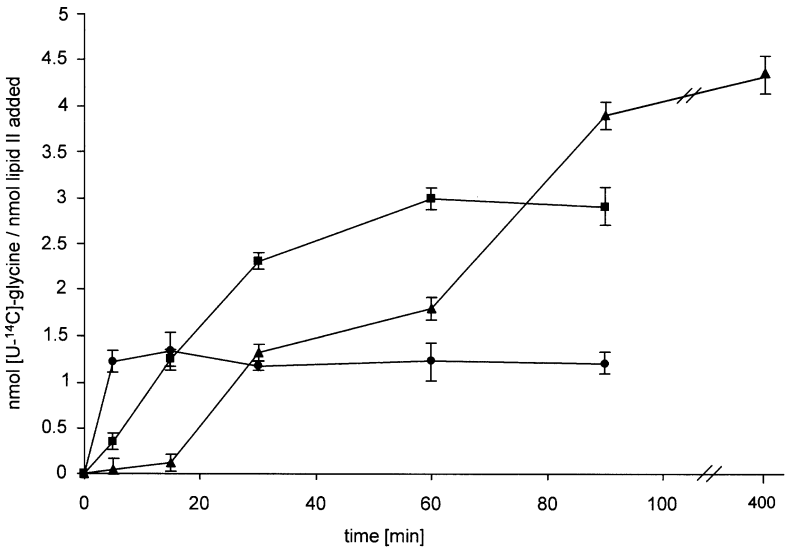

Fig. 7. Time dependency of the FemXAB reactions. Purified lipid II was incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA synthetase and tRNA with addition of FemX (circles), FemX and FemA (squares) and FemX, FemA and FemB (triangles); mean values of three experiments are given.

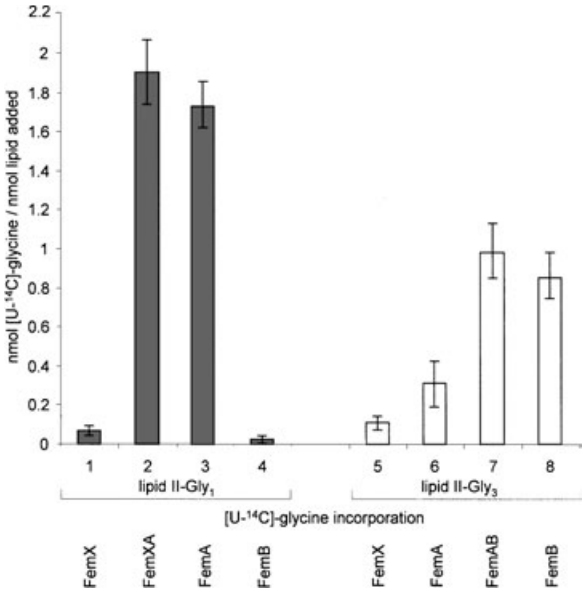

Fig. 8. Analysis of the FemA and FemB reaction using purified lipid II-Gly_1 (grey columns) and lipid II-Gly_3 (white columns). Purified FemX product (grey) and FemXA product (white) were incubated with [U-^{14}C]-glycine in the presence of recombinant tRNA-synthetase and purified tRNA with FemX (columns 1 and 5), FemX and FemA (column 2), FemA only (columns 3 and 6), FemA and FemB (column 7) and FemB only (columns 4 and 8); mean values of two experiments are given.

targets as inhibition restores susceptibility towards methicillin (Labischinski, 1992; Ling and Berger-Bächi, 1998). Therefore, we established an *in vitro* system using purified bactoprenol-bound and soluble cell wall precursors as well as His-tagged enzymes to allow detailed analysis of single steps of glycine addition. Additionally, we included purified staphylococcal tRNAs and Gly-tRNA-synthetase from *S. aureus* in the synthesis assay as the glycine donors for interpeptide synthesis are non-proteinogenic Gly-tRNAs (Matsuhashi *et al*., 1967). Interestingly, in the genome of *S. aureus*, four or five different Gly-tRNAs are present, two proteinogenic and three non-proteinogenic (Green and Vold, 1993), leading to the hypothesis that each Fem factor may use dedicated Gly-tRNAs, which remain to be attributed. In spite of the specialization at the tRNA level, *S. aureus* disposes of only one Gly-tRNA-synthetase to charge both proteinogenic and cell wall-associated Gly-tRNAs in an ATP-dependent fashion (Niyomporn *et al*., 1968). In contrast to earlier studies (Kamiryo and Matsuhashi, 1972), we could not observe glycine incorporation using tRNA-synthetase and tRNAs isolated from *Escherichia coli* when used in combination with *S. aureus* tRNAs or tRNA-synthetase respectively (data not shown).

In the *in vitro* system, neither the soluble UDP-MurNAc-pentapeptide nor lipid I was converted to glycine-labelled precursors by the FemABX peptidyltransferases. Only the complete lipid II molecule could serve as substrate for FemX and the respective Gly additives subsequently for FemA and FemB. Therefore, we may conclude that FemX should recognize lipid II via the sugar moiety, i.e. GlcNAc-MurNAc. By analogy, FemA and FemB may also recognize lipid II via the disaccharide unit. This could be an explanation for the reduced reaction rate of FemX in the presence of FemA (see also below). In addition, structural data for FemA obtained from X-ray analysis with a resolution at 2.1 Å identified a putative, L-shaped binding groove, which could accommodate the disaccharide unit (Benson *et al*., 2002b).

The single steps of glycine addition by the Fem proteins were analysed by incorporation of radiolabelled glycine to lipid II. The substrate was completely converted to lipid II-Gly_1 by FemX within 5 min, which corresponds to a turnover rate of 19 lipid II molecules per FemX molecule min^{-1}. (The ratio of FemX:lipid II in the *in vitro* assay was ≈1:100.) Such a turnover rate is in the same order of magnitude as that calculated recently for Gram-negative bacteria. Mengin-Lecreux and van Heijenoort (1985) calculated that, in *E. coli* K-12, 24 disaccharide peptide units need to be transported per min per bactoprenol carrier (3.5×10^6 disaccharide peptide units per cell; generation time 36 min; 50% turnover per generation; about 2000 lipid II molecules per cell). It is not known whether the turnover of lipid II in Gram-negative and Gram-positive bacteria is similar. The amount of peptidoglycan per dry weight differs greatly with about 10% in Gram-negative and about 30–70% in Gram-positive bacteria. However, when compared with *E. coli*, the number of lipid II molecules in staphylococci is 25 times higher than that determined for *E. coli* (Somner and Reynolds, 1990), leading to the assumption that synthesis of the cell wall in Gram-positive bacteria depends on the amount of lipid II rather than on an increased synthesis rate.

A rate-limiting factor for the FemABX reaction *in vitro* was the availability of glycine donor tRNAs. Although increasing concentrations of tRNAs lead to increasing amounts of incorporated glycine, the FemABX reaction never reached the expected values of Gly_5. Similarly, amino acid analysis of peptidoglycan synthesized *in vivo* revealed submaximal values of incorporated glycine, reflecting a minor presence of Gly_3 and Gly_1 interpeptide bridges (Stranden *et al*., 1997; Tschierske *et al*., 1997; Roos *et al*., 1998; Ehlert *et al*., 2000a). In addition, synthesis of a complete lipid II-Gly_5 may be favoured in the *in vivo* situation with ongoing translocation of the completely modified cell wall precursor to the outside of the cell.

As confirmed by mass spectrometry, the *in vitro* FemX product is lipid II-Gly_1, demonstrating that FemX indeed adds the first Gly to the pentapeptide side-chain. When

lipid II was incubated with FemXA, only lipid II-Gly_3 was found but no lipid II-Gly_2 (Fig. 3.1). This observation agrees with the hypothesis that FemA adds two glycine molecules per cycle, not releasing a lipid II-Gly_2 product, whereas FemX clearly adds only one Gly per cycle to the lipid intermediates. The molecular basis for such a difference in the catalytic mechanism has not yet been resolved.

The FemA protein presumably contains just one Gly-tRNA binding site, which led Benson *et al*. (2002) to the hypothesis that, after the addition of the first Gly, a subsequent round of Gly-tRNA binding and transfer would account for the addition of the second glycine. However, recently, direct interactions of FemA–FemA, FemB–FemB and FemA–FemB were detected using the bacterial two-hybrid system. FemX did not show any interactions with FemA or FemB. This rather suggested that FemX acts as a monomer while FemA and FemB may act as homodimers (Rohrer and Berger-Bächi, 2003b). Interestingly, FemA and FemB were also found to interact with the lysostaphin immunity factor Lif of *S. simulans* biovar *staphylolyticus*, which was shown to substitute serine in positions 3 and 5 of the pentaglycine interpeptide chain in *S. aureus* in co-operation with FemA or FemB (Thumm and Götz, 1997; Ehlert *et al*., 2000). These data imply that FemA and FemB probably function as homodimers in such a way that both subunits are loaded with Gly-tRNA to ensure sequential addition of both Gly to the growing interpeptide bridge. Furthermore, addition of Ser at the defined positions 3 and 5 upon interaction with FemA or FemB leads to the assumption that recognition of the lipid-bound substrate is mediated by the Fem peptidyltransferases, whereas Lif may not possess an appropriate binding groove.

In this study, the FemX reaction was retarded upon addition of FemA and further impaired when FemB was also present. As described above, inhibition by protein–protein interactions of FemX with FemA and/or FemB seems unlikely. Rather, the retarded conversion of lipid II by FemX in the presence of FemAB may be caused by competitive, non-productive binding of lipid II by FemA and FemB. Correspondingly, the non-productive binding by FemX and FemB could delay the formation of lipid II-Gly_3, and binding by FemX and FemA the formation of lipid II-Gly_5 respectively. If so, FemAB must recognize lipid II via the disaccharide unit regardless of the amount of Gly added. However, the appropriate number of Gly in the interpeptide bridge is clearly required for FemAB to be active, i.e. for FemA at least one and for FemB at least three Gly need to be present.

Obviously, the enzymatic activity of an individual FemXAB peptidyltransferase does not require the presence of a second species, and the formation of a stable Fem–multienzyme complex is not necessary to achieve a turnover rate *in vitro* that is close to the *in vivo* situation. However, *in vivo*, production of byproducts as observed during *in vitro* synthesis upon increased incubation time and elevated enzyme concentration must be prevented, and it seems reasonable to assume that the interpeptide bridge formation is highly co-ordinated, tightly interlinked with the other membrane-associated steps of cell wall biosynthesis (Fig. 9). Possibly, the Fem proteins are transiently associated with each other as well as with other cell wall biosynthesis enzymes. Moreover, further components with as yet unidentified functions may contribute to co-ordinating the intracellular and extracellular processes to ensure the proper formation of the vital bacterial cell wall.

Experimental procedures

Chemicals and materials

All chemicals were of analytical grade or better. *E. coli* tRNA and *E. coli* tRNA-synthetase, UDP-*N*-acetyl-glucosamine (UDP-GlcNAc) were purchased from Sigma.

In vitro *lipid I/lipid II synthesis and purification*

Synthesis and purification was performed according to the protocol elaborated by Brötz *et al*. (1995) for [^{14}C]-lipid II with modifications by Breukink *et al*. (2003). In short, lipid II was synthesized *in vitro* using membrane preparations of *Micrococcus flavus* DSM 1790. Membranes were isolated from lysozyme-treated cells by centrifugation (40 000 *g*), washed twice in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5, and stored under liquid nitrogen until use. The analytical assay was performed in a total volume of 150 µl containing 400 µg of membrane protein, 10 nmol of undecaprenylphosphate, 100 nmol of UDP-*N*-acetylmuramic acid pentapeptide (UDP-MurNAc-PP), 100 nmol of UDP-GlcNAc in 60 mM Tris-HCl, 5 mM $MgCl_2$, pH 8, and 0.5% (w/v) Triton X-100. UDP-MurNAc-PP was purified as described previously (Kohlrausch and Höltje, 1991). For purification of milligram quantities of lipid II, the analytical procedure was scaled up by a factor of 250. Reaction mixtures were incubated for 1 h at 30°C, and lipids were extracted with the same volume of n-butanol/6 M pyridine-acetate, pH 4.2. Lipid I was synthesized as described for lipid II with the omission of UDP-GlcNAc from the synthesis reaction. Purification of lipid I/lipid II was performed on a DEAE-cellulose column (0.9 × 25 cm, DEAE SS-Typ; Serva) and eluted in a linear gradient from chloroform–methanol–water (2:3:1) to chloroform–methanol–300 mM ammonium bicarbonate (2:3:1). Lipid I- and lipid II-containing fractions were identified by TLC (silica plates, 60F254; Merck) using chloroform–methanol–water–ammonia (88:48:10:1) as the solvent (Rick *et al*., 1998). Spots were visualized by PMA staining reagent. The concentration of purified lipids was determined as inorganic phosphate after treatment with perchloric acid (Rouser *et al*., 1970).

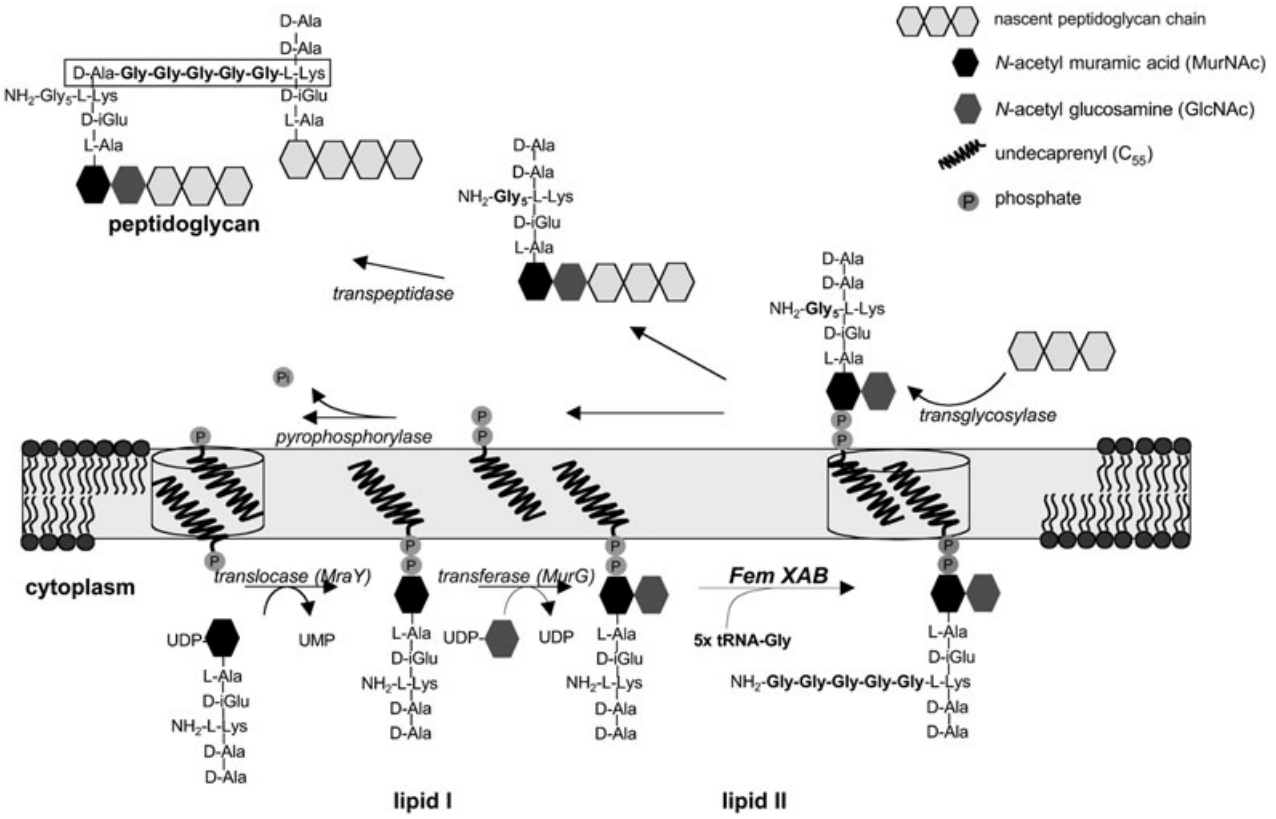

Fig. 9. Cell wall biosynthesis of *S. aureus*. Formation of the pentaglycine interpeptide bridge does not occur before synthesis of the cell wall precursor lipid II is completed. When the interpeptide bridge is accomplished, the monomeric peptidoglycan unit is translocated across the cytoplasmic membrane to the outside and incorporated into the cell wall. UMP, uridine monophosphate; UDP, uridine diphosphate.

Preparation of membranes and supernatants from S. aureus *NCTC 8325 and* S. simulans *22 for the synthesis of lipid–glycine intermediates*

Staphylococcus aureus NCTC 8325 and *S. simulans* 22 cells were grown in 1.2 l of TSB medium (Merck) at 37°C to an OD_{600} of 4–5, harvested by centrifugation (10 min, 8000 *g*) and washed once with 50 mM Tris-HCl buffer, 10 mM $MgCl_2$, pH 7.5. Cells were resuspended in 20 ml of the same buffer and lysed by grinding with glass beads (diameter 0.1 mm, B. Braun; 20 ml of beads was added to 20 ml of cell suspension). After centrifugation (30 min, 40 000 *g*), the membrane fraction and supernatant fraction (supernatant I) were frozen in liquid nitrogen and stored at −70°C until use. Supernatant II was obtained after high-speed centrifugation at 100 000 *g* for 30 min. Protein concentrations were determined using the BCA assay (Pierce) (Smith *et al*., 1985).

Construction of the GlyS-His$_6$ vector

Staphylococcus aureus NCTC 8325 glycyl-tRNA-synthetase gen *glyS* was amplified with specific primers containing an *Nhe*I (GCTAGC) site or a *Xho*I (CTCGAG) site (*glyS*$^+$: 5′-CTAGCTAGCGCAAAAGATATGGATAC-3′; *glyS*$^-$: 5′-CATCTCGAGGAATTTTGTTTTTTCAGTTAAG-3′). Polymerase chain reaction (PCR) products and pET24b vector (Novagen) were digested by *Nhe*I–*Xho*I for ligation.

The cloning strategy resulted in Met-Ala-Ser and Leu-Glu-$(His)_6$ as N- and C-terminal sequences of the glycyl-tRNA-synthetase.

Overexpression of His$_6$-tagged proteins

Escherichia coli BL21 (DE3) cells transformed with the recombinant plasmid were grown in Luria–Bertani medium at 30°C. At an optical density (OD_{600}) of 0.6, IPTG was added to a concentration of 0.5 mM to induce expression of the recombinant proteins. After 1.5 h, cells were harvested and resuspended in lysis buffer (50 mM Na_2HPO_4, pH 7.8, 300 mM NaCl, 10 mM imidazole). Aliquots of 200 μg ml^{-1} lysozyme, 100 μg ml^{-1} DNase and 10 μg ml^{-1} RNase were added; cells were incubated for 30 min on ice and sonicated. Cell debris was spun down, and the supernatant was added to 1 ml of Ni-NTA-agarose slurry (Qiagen). This mixture was gently turned upside down at 4°C for 1 h and then loaded on to a column support. After washing with lysis buffer, weakly bound material was removed with 50 mM Na_2HPO_4, pH 7.8, 300 mM NaCl and 20 mM imidazole. His-tagged proteins were eluted with buffer containing 50 mM Na_2HPO_4, pH 7.8, 300 mM NaCl and 200 mM imidazole. Three fractions were collected, which were stored in 50% glycerol at −20°C. Pro-

tein concentrations were determined using the Bradford method (Bradford, 1976) with BSA as a standard. Purity was controlled by SDS-10% PAGE, and fractions containing 90% fusion protein were used for further experiments.

Isolation of tRNA from S. aureus *NCTC 8325 and* S. simulans *22*

Cells were grown in 3 l of Mueller–Hinton broth at 37°C to an OD_{600} of 0.7 and harvested. Bacteria were resuspended in 20 ml of water, added to 50 ml of boiling water and boiled for 15 min. Insoluble material was removed by centrifugation (30 min, 40 000 *g*). The bacterial lysate was lyophilized and resuspended in 10 ml of RNase-free water. Small RNA molecules were purified further using the DNA/RNA purification kit from Qiagen according to the instructions given by the manufacturer; purity was controlled on 0.8% agarose gels. Purified tRNA was stored at –20°C and heated to 65°C for 3 min before use.

In vitro *synthesis of glycine-labelled lipid intermediates*

The assays for synthesis of glycine-labelled lipid intermediates were performed in a total volume of 100 μl containing 300–350 μg of protein of membranes and/or supernatant and the substrates for lipid II synthesis [10 nmol of undecaprenylphosphate, 100 nmol of UDP-GlcNAc, 100 nmol of UDP-MurNAc-PP in 100 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5, and 0.8% Triton X-100, 2 mM ATP and 50 nmol of [U-^{14}C]-glycine (3.7 GBq mmol^{-1}; Amersham Pharmacia Biotech)]. If not indicated otherwise, the synthesis mixtures contained 5 nmol of lipid I, lipid II or lipid intermediates, 300–350 μg of supernatant protein (or 10 μg of tRNA-synthetase and 25 μg of tRNA), 2 mM ATP and 50 nmol of [U-^{14}C]-glycine in 100 μl of buffer (see above). Purified Fem proteins were added at a concentration of 2.7 μg per 100 μl. Reaction mixtures were incubated for 60 min at 30°C except for the complete ABX reaction (17 h) or as indicated. Lipid intermediates were extracted from the reaction mixture with the same volume of n-butanol/pyridine acetate, pH 4.2, and analysed by TLC (see above). Radiolabelled spots or lanes were visualized by iodine vapour, excised and quantified by β-scintillation counting (1900 CA Tri-Carb scintillation counter; Packard). Isolation of larger quantities of non-radioactive-labelled Gly–lipid II intermediates was achieved with an upscale of the synthesis assay applying the purification protocol described for lipid II.

Mass spectrometry

Electrospray MS spectra were recorded on an API-I single quadrupole instrument (PE-SCIEX) working in positive mode. Samples were infused at 100–200 μl h^{-1}, either directly (in methanol–chloroform, 1:1) or diluted 1:1 with 0.5% formic acid in methanol. Sample concentrations ranged from 5 to 20 nmol per 100 μl. A needle voltage of 5600 V and an orifice voltage of 90–120 V were used, and spectra accumulated for at least 100 scans. Signals from the monocharged species (m^+H^+) were accompanied in all cases by those from the monosodium adduct, the monopotassium adduct and, in some cases, the disodium adduct. In general, the magnitude of the signal decreased with increasing glycination.

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Role of σ^B in the Expression of *Staphylococcus aureus* Cell Wall Adhesins ClfA and FnbA and Contribution to Infectivity in a Rat Model of Experimental Endocarditis

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Isogenic *Staphylococcus aureus* strains with different capacities to produce σ^B activity were analyzed for their ability to attach to fibrinogen- or fibronectin-coated surfaces or platelet-fibrin clots and to cause endocarditis in rats. In comparison to the σ^B-deficient strain, BB255, which harbors an *rsbU* mutation, both *rsbU*-complemented and σ^B-overproducing derivatives exhibited at least five times greater attachment to fibrinogen- and fibronectin-coated surfaces and showed increased adherence to platelet-fibrin clots. No differences in adherence were seen between BB255 and a Δ*rsbUVWsigB* isogen. Northern blotting analyses revealed that transcription of *clfA*, encoding fibrinogen-binding protein clumping factor A, and *fnbA*, encoding fibronectin-binding protein A, were positively influenced by σ^B. σ^B overproduction resulted in a statistically significant increase in positive spleen cultures and enhanced bacterial densities in both the aortic vegetations and spleens at 16 h postinoculation. In contrast, at 72 h postinoculation, tissues infected with the σ^B overproducer had lower bacterial densities than did those infected with BB255. These results suggest that although σ^B appears to increase the adhesion of *S. aureus* to various host cell-matrix proteins in vitro, it has limited effect on pathogenesis in the rat endocarditis model. σ^B appears to have a transient enhancing effect on bacterial density in the early stages of infection that is lost during progression.

Staphylococcus aureus is a major human pathogen with the capacity to cause a broad spectrum of diseases including native and prosthetic valve endocarditis (34). Its ability to colonize vascular tissue is thought to occur via ligand-adhesin interactions between the organism's surface determinants and host proteins at sites of endovascular injuries and prosthetic materials (8, 11, 14, 40). *S. aureus* expresses a variety of distinct surface proteins, termed MSCRAMMs (for "microbial surface components recognizing adhesive matrix molecules"), which allow the pathogen to bind to host extracellular matrix proteins and establish a focus of infection (17, 21, 38, 43). Among the MSCRAMMs are the clumping factors ClfA and ClfB (35, 36, 37, 42), which modulate bacterial adhesion to fibrinogen, and the fibronectin-binding proteins FnbA and FnbB, which mediate binding to fibronectin (15, 16, 17, 26) and to a lesser extent fibrinogen (53). Fibrinogen is a large (340-kDa) protein found predominantly in blood. It is also the most abundant host protein in endothelial lesions (13), as well as the major blood protein deposited on newly implanted biomaterials (52). Fibronectin is a dimeric glycoprotein, which forms a significant component of the conditioning layer that coats many implanted medical devices such as heart valves and catheters. Both the clumping factors and the fibronectin-binding proteins influence the pathogenicity of *S. aureus* in various infection models (14, 32, 40, 44, 46, 48, 52). It has been shown previously that σ^B significantly influences the clumping of *S. aureus* in the presence of soluble fibrinogen or fibronectin in vitro (3, 41).

In the present study, we used a series of isogenic strains to characterize the effects of σ^B activity on the capacity of *S. aureus* to adhere to immobilized fibrinogen, fibronectin, and platelet-fibrin clots. Additionally, we examined the effects of σ^B on *S. aureus* infectivity in a rat model of experimental endocarditis (14, 22, 40).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and relevant phenotypes are listed in Table 1. Microorganisms were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) or Luria-Bertani medium, with aeration at 37°C and 200 rpm. For both in vitro adhesion experiments and in vivo inoculum preparation, overnight cultures of bacteria were diluted 1:100 in fresh medium and grown to an optical density at 600 nm of 2.0, where σ^B was shown to be highly active (2). Where indicated, the media were supplemented with 5 μg of erythromycin per ml or 10 μg of tetracycline per ml. The presence of antibiotics did not alter the adherence phenotype in vitro, and the mutants grew as well as the parental strain did.

Northern blot analyses. Total RNA was isolated as described by Cheung et al. (7). An 8-μg portion of total RNA from each sample was electrophoresed through a 1.5% agarose–0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA [pH 7]). RNA was blotted onto a positively charged nylon membrane (Roche, Basel, Switzerland) with a vacuum blotter (Pharmacia, Uppsala, Sweden). The intensity of the 23S and 16S rRNA bands stained with ethidium

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Source or reference
Strains		
E. coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F^+ *proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tc^r)]	Stratagene
S. aureus		
BB255	*rsbU;* low σ^B activity	1
IK181	BB255 Δ*rsbUVWsigB;* Em^r	30
GP268	BB255 *rsbU*$^+$; Tc^r	19
MB138	BB255 *rsbW7* (Am); Em^r (high level of σ^B activity)	2
DU5880	8325-4 *clfA1*::Tn*917;* Em^r	35
Plasmids		
pAC7	Cm^r, expression plasmid containing the P_{BAD} promoter and the *araC* gene	45
pAC7-*sigB*	Cm^r, 767-bp PCR fragment of the *sigB* ORF from strain COL into pAC7	23
pSB40N	Ap^r, promoter probe plasmid	29
pSB40N*clfA*p	Ap^r, 500-bp chromosomal fragment covering the *clfA* promoter region of COL into pSB40N	23
pSB40N*fnbA*p	Ap^r, 250-bp PCR fragment covering the *fnbA* promoter region of BB255 into pSB40N	This study

[a] Abbreviations: Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Em^r, erythromycin resistant; Mc^r, methicillin resistant; Tc^r, tetracycline resistant; ORF, open reading frame.

bromide was verified to be equivalent in all the samples before transfer. Labeling and hybridization were done by using the digoxigenin labeling and detection kits as specified by the manufacturer (Roche). The following primers were used to generate the digoxigenin-labeled DNA probes by PCR labeling: *clfA* for, 5′-CG ATTGGCGTGGCTTCAGTGC-3′; *clfA* rev; 5′-CGTTGTTGAAACATTTTCC GC-3′ (nucleotides 345 to 365 and 806 to 826 of accession no. Z18852); *fnbA* for, 5′-GGGATGGGACAAGATAAAGAAGC-3′; *fnbA* rev., 5′-ACGACACGTTG ACCAGCATG-3′; (nucleotides 174924 to 174902 and 174366 to 174347 of accession no. AP003137.2).

Construction of plasmid pSB40N*fnbAp*. For pSB40N*fnbAp*, 250 bp of the *fnbA* promoter region of BB255 was synthesized by PCR using the upstream primer 5′-GCGGATCCGTTTTAAATTAGATAATGATG-3′ (the BamHI linker is underlined) and the downstream primer 5′-CGCCTCGAGCCCTTTAAATGCAA AATTCATT-3′ (the XhoI linker is underlined). The PCR product was digested with BamHI and XhoI and cloned into promoter probe plasmid pSB40N (29) to obtain pSB40N*fnbAp*. Sequence analysis confirmed the identity of the insert. Plasmid pSB40N*fnbAp* was subsequently transformed into *Escherichia coli* XL1Blue containing either plasmid pAC7-*sigB* or pAC7 (23). The two-plasmid testing was performed as described by Homerova et al. (23).

Functional expression of fibrinogen and fibronectin activity. Adherence of *S. aureus* cells to solid-phase fibrinogen or fibronectin was measured by two methods. In the first method, flat-bottom microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with serial dilutions (in phosphate-buffered saline PBS) of either human fibrinogen (Sigma, Buchs, Switzerland) or fibronectin (Sigma) and blocked with 2 mg of bovine serum albumin per ml in phosphate-buffered saline (PBS). Then 50 μl of bacterial cells, corresponding to 5×10^8 CFU, was added to each well, and the mixtures were incubated at 37°C for 2 h. To monitor the effects of proteases on *S. aureus* adherence, cells were precultured and assayed in either the presence or absence of 0.4 U of the global protease inhibitor α_2-macroglobulin (Roche Diagnostics, Rotkreuz, Switzerland) per ml. Adherent cells were fixed with warm air (60°C) for 30 min and stained with 0.5% (wt/vol) crystal violet for 45 min. After addition of 200 μl of citrate buffer (pH 4.3) and incubation for 45 min at room temperature, the absorbances were read in an enzyme-linked immunosorbent assay reader at 570 nm. Each batch of assays included at least one control strain as well as blank wells.

In a second assay the fibrinogen- and fibronectin-binding capacities of the BB255 derivatives were determined by direct binding of washed cells (10^4 CFU) to solid-phase fibrinogen and fibronectin in six-well plates by using a method previously described by Kupferwasser et al. (31).

FACS analysis. The abundance of ClfA on the cell surface was measured by fluorescent-activated cell sorter (FACS) analysis using purified $F(ab')_2$ fragments from anti-ClfA rabbit polyclonal immunoglobulin G (kindly provided by T. Foster, Dublin, Ireland) as described previously (49). Briefly, *S. aureus* cells were washed, suspended in PBS containing 10% fetal calf serum-antibody solution (10 μg of antibody/ml), and incubated for 2 h at 4°C. The cells were then washed twice with PBS–10% fetal calf serum, suspended in PBS–10% fetal calf serum-antibody solution containing $F(ab')_2$ fragments of a fluorescein isothiocyanate-labeled swine anti-rabbit antibody (DakoCytomation, Glostrup, Denmark), incubated for 1 h at 4°C, washed, fixed with 4% formaldehyde solution for 30 min at 4°C, and resuspended in PBS–10% fetal calf serum before being subjected FACS analysis. *S. aureus* DU5880 (35), a *clfA*-negative strain, was used as control.

Attachment to human platelet-fibrin clots in vitro. Platelet-fibrin clots were produced by pouring 0.5 ml of plasma into 30-mm-diameter cell culture plates (Corning Coster, Corning, N.Y.) in the presence of 100 μl of bovine thrombin solution (5,000 National Institute of Health units per ml) (Diagnotec AG, Liestal, Switzerland) and 100 μl of a 0.2 mM $CaCl_2$ solution. The clots were dehydrated overnight at 30°C and kept at 4°C until used. Bacterial adherence was determined by adding 2 ml of saline containing 10^4 CFU per ml to the wells and agitating the plates for 3 min at 120 rpm on a rotating incubator. After gentle decantation of the fluid, the clots were washed twice for 5 min with 2 ml of PBS to remove nonadherent bacteria. They were then overlaid with 3 ml of Columbia agar, which was allowed to solidify prior to incubation at 37°C. The number of adherent bacteria that gave rise to colonies was determined after 24 h of incubation and expressed as a multiple of adherent organisms relative to the original inoculum (adherence ratio = [number of adherent bacteria/inoculum size] × 10^4). The statistical significance of the different attachment of the test organisms to platelet-fibrin clots was evaluated by the one-way analysis of variance with Bonferroni's correction for multiple group comparisons.

Experimental endocarditis. Catheter-induced vegetations of the aortic valve were produced in rats as previously described (22, 40). Groups of animals were inoculated 24 h after catheterization by intravenous injection of 0.5 ml of saline containing increasing numbers of organisms from the late exponential growth phase. The animals were sacrificed 16 h after the bacterial challenge, and quantitative vegetation and spleen cultures were performed. Vegetation and spleen tissues were serially diluted and plated onto both antibiotic-free tryptic soy agar plates and tryptic soy agar plates containing the appropriate antibiotic, allowing characterization of the stability of all mutants after animal passage. In a second set of experiments, animals were sacrificed 72 h after the bacterial challenge and vegetation and spleen cultures were processed as described above. Statistical differences comparing the rates of aortic valve and spleen infections were evaluated by the χ^2 test with the Yates correction. Differences between median bacterial densities in infected tissues were analyzed by the Mann-Whitney test. Data were considered significant when P values were <0.05 by the use of two-tailed significance levels.

RESULTS AND DISCUSSION

Effect of σ^B on *clfA* and *fnbA* expression. Microarray-based analysis of the transcriptional profiles of three genetically dis-

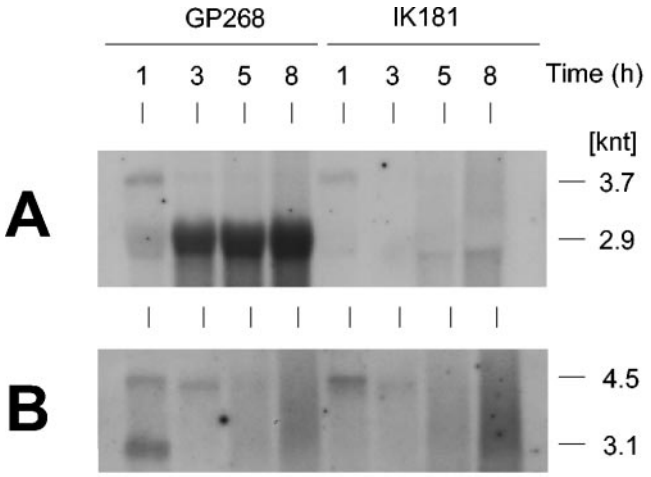

FIG. 1. Northern blot analyses of *clfA* and *fnbA*. Total RNAs of strains GP268 (BB255 *rsbU*$^+$) and IK181 (BB255 Δ*rsbUVWsigB*) grown in LB medium and harvested at the time points indicated were probed with *clfA* (A) and *fnbA* (B), respectively. Relevant transcripts and their sizes are indicated.

tinct *S. aureus* strains indicated that *clfA* and *fnbA* were positively influenced by σ^B activity (4). In those studies, *clfA* transcription appeared to be strongly up-regulated by σ^B during later growth stages whereas no significant differences in *clfA* expression were observable during early growth. We confirmed these findings by Northern blot analyses. As shown in Fig. 1, the *clfA* transcript was produced in a σ^B-dependent manner. A 2.9-knt transcript was strongly produced in the *rsbU*-complemented strain, GP268, but was very weak in the isogenic Δ*rsbUVWsigB* mutant, IK181 (Fig. 1A). In addition to the σ^B-dependent transcript, a larger *clfA*-encoding transcript (4.5 knt) was observed during early growth in both GP268 and IK181, suggesting that basal transcription of *clfA* is governed in *S. aureus* by a σ^B-independent process during early growth stages. The finding that *clfA* expression increased with growth is striking and contradicts the common paradigm that MSCRAMM-encoding genes are expressed predominantly during the early growth stages.

In contrast to *clfA*, microarray analysis (4) indicated that the presence of a functional *sigB* operon promotes *fnbA* transcription mainly during the early growth stages but not in later stages. Northern blot analysis identified two distinct *fnbA*-encoding transcripts (Fig. 1B), with the shorter transcript (3.1 knt) being σ^B dependent and the larger transcript (3.7 knt) being independent of σ^B activity. The shorter transcript was observed only in GP268 during early growth, while the larger transcript was detectable in both BB255 derivatives analyzed until mid-log phase, corroborating the microarray results (4).

Two-plasmid testing of the *clfA* and *fnbA* promoter regions. We used a two-plasmid system, which has proved useful in identifying promoters recognized by RNA polymerase containing *S. aureus* σ^B (23), to test whether the *fnbA* promoter region that gives rise to the 3.1-knt transcript is recognized by σ^B. The *clfA* promoter, which was already identified to be σ^B dependent by this method, was used as positive control (23). *E. coli* strains harboring plasmids pAC7*sigB* and pSB40N*clfAp* yielded dark blue colonies upon selection on LBACX-ARA plates, confirming that there is a physical interaction between the *S. aureus* σ^B-*E. coli* RNA polymerase holoenzyme hybrid and the *clfA* promoter region. However, no such interaction was observable for the *E. coli* transformant harboring plasmids pAC7*sigB* and pSB40N*fnbAp*, suggesting that σ^B does not directly mediate *fnbA* transcription. Interestingly, *E. coli* cells harboring pSB40N*fnbAp* and the control plasmid, pAC7, yielded light blue colonies on LBACX- ARA plates, indicating that the *fnbA* promoter was recognized by the σ^A-containing *E. coli* RNA polymerase holoenzyme. These findings, together with the transcriptional profile of *fnbA* during in vitro growth (4) and the lack of a significant σ^B consensus promoter sequence (23) in the upstream region of the *fnbA* open reading frame, strongly suggest that the positive effect of σ^B on *fnbA* transcription is most probably indirect and occurs by an as yet unidentified mechanism. It is known that *fnbA* transcription is enhanced by SarA (12,54, 55) and down-regulated by *agr* (47, 55). It has been suggested that σ^B activity has a positive effect on the expression of the *sarA* locus (3, 4, 18), as well as a negative effect on the expression of the *agr* locus (3, 4, 24). Thus, the two global regulators may, at least in part, account for the up-regulatory effect of σ^B activity on *fnbA* transcription. However, the mechanisms by which σ^B modulates SarA production are not completely understood (3, 4, 9, 10, 18, 24).

σ^B dependence of surface-bound ClfA. To determine whether the σ^B-dependent increase in *clfA* expression correlates with an increase in the amount of surface-bound ClfA, we used FACS analysis to determine the ClfA abundance on the surfaces of a series of BB255 derivatives with differing abilities to produce σ^B activity. As indicated in Fig. 2, FACS shifts were clearly observed for the *rsbU*-complemented strain, GP268 (Fig. 2C), and the BB255 derivative, MB138 (Fig. 2D), with high σ^B activity due to an amber mutation within the gene encoding the anti-σ^B factor RsbW (2). In contrast, BB255 (Fig. 2A) and its Δ*rsbUVWsigB* mutant, IK181 (Fig. 2B) demonstrated very slight FACS shifts. A *clfA*-negative *S. aureus* strain, DU5880, was negative by FACS analysis (data not shown), indicating that the shifts observed for the BB255 derivatives were ClfA specific.

Functional expression of fibrinogen and fibronectin activity. The findings that *clfA* and *fnbA* expression are positively influenced by σ^B raises the question whether and how σ^B affects *S. aureus* adherence to the host cell matrix proteins fibrinogen and fibronectin. The BB255 derivatives were therefore analyzed for their abilities to attach to fibrinogen- or fibronectin-coated surfaces in vitro (Fig. 3). GP268 and MB138 exhibited at least five times greater attachment to either fibrinogen-coated (Fig. 3A) or fibronectin-coated (Fig. 3B) surfaces than BB255 did. Moreover, the binding capacities correlated with σ^B activity; the greatest binding was observed with the σ^B overproducer, MB138, and the lowest binding was observed with the Δ*rsbUVWsigB* mutant IK181. No obvious differences in adherence were detectable between BB255 and IK181, corroborating previous findings suggesting that BB255, although producing a significant amount of σ^B, is unable to activate this alternative transcription factor due to its inability to generate the positive regulator of σ^B activity, RsbU (3, 19).

Similar results were observed when bacterial binding to solid fibrinogen and fibronectin was determined by a method described by Kupferwasser et al. (31). The numbers of CFU

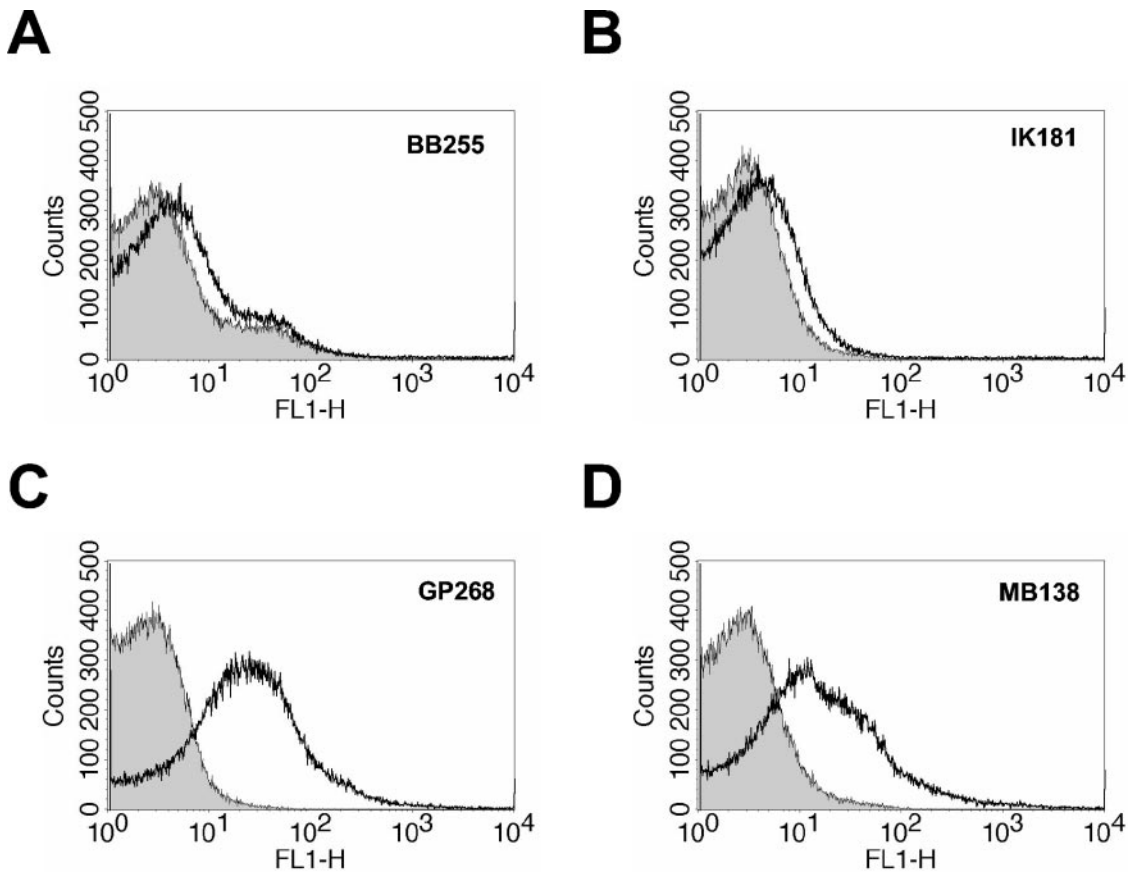

FIG. 2. Histograms of ClfA expression on the surface of *S. aureus* cells. FACS analysis was performed with both irrelevant antibodies (gray areas) and purified anti-ClfA F(ab′)$_2$ fragments (white areas). (A) BB255 (*rsbU*); (B) IK181 (BB255 Δ*rsbUVWsigB*); (C) GP268 (BB255 *rsbU*$^+$); (D) MB138 [BB255 *rsbW7*(Am)].

bound to solid fibrinogen and fibronectin were again significantly increased ($P < 0.05$) in both GP268 and MB138 compared to those in BB255 and IK181 (Fig. 4). Both experimental outcomes are consistent with previous findings showing a positive effect of σ^B on the attachment of *S. aureus* to either soluble fibrinogen (41) or soluble fibronectin (3).

Effect of the universal protease inhibitor α_2-macroglobulin on the adherence phenotype of the BB255 derivatives. Karlsson et al. (27) reported that in *sarA* mutant cells, which produced large amounts of the four major extracellular proteases of *S. aureus*, the levels of cell-bound fibronectin-binding proteins A and B were low in comparison to those in wild-type cells. This was found to occur independently of *fnbA* or *fnbB* transcription. More recently, the same authors showed that extracellular protease production among clinical isolates of *S. aureus* varied mainly due to differences in *sarA* expression. They further indicated that the level of σ^B-dependent expression of *sarA* determined the level of protease production; there was an inverse correlation between σ^B activity and protease activity (28). Because synthesis of extracellular proteases is activated by *agr* (5, 25, 33) and because *agr* expression is negatively regulated by σ^B activity (3, 4, 24), it is reasonable that σ^B activity down-regulates extracellular protease production in an indirect manner. This σ^B-mediated decrease in extracellular protease levels is likely to increase the amount of wall-anchored fibronectin-binding protein and thus may be, at least for fibronectin, the primary reason for the observed increase in the adherence of *sigB*$^+$ strains. To determine whether σ^B-SarA-mediated differences in protease activity might account for the adherence differences observed between *sigB*$^+$ and *sigB* mutant strains, we repeated the adherence experiments in the presence of the universal protease inhibitor α_2-macroglobulin. The fibrinogen and fibronectin adherence profiles obtained in presence of α_2-macroglobulin were almost identical to those shown in Fig. 3. This finding does not agree with the recent findings by Xiong et al. (55), who observed a 30% increase in fibronectin-binding activity for strains grown in the presence of α_2-macroglobulin compared to strains grown in its absence. However, Xiong et al. noted that the predominant mechanism dictating phenotypic fibronectin binding seems to occur at the level of *fnbA* transcription. Our findings support this hypothesis and indicate that the differences in adherence observed between *sigB*$^+$ and *sigB* mutant strains were not likely to be due to σ^B-SarA-mediated alterations in the protease activity of the respective strains but were caused by the increased σ^B-dependent expression of *clfA* and *fnbA*. Furthermore, fibrinogen- and fibronectin-binding protein homologues, such as ClfB and FnbB, whose expression is not considered to be affected by σ^B activity, were apparently not able to mask the strong decrease in adherence to fibrinogen and fibronectin observed for

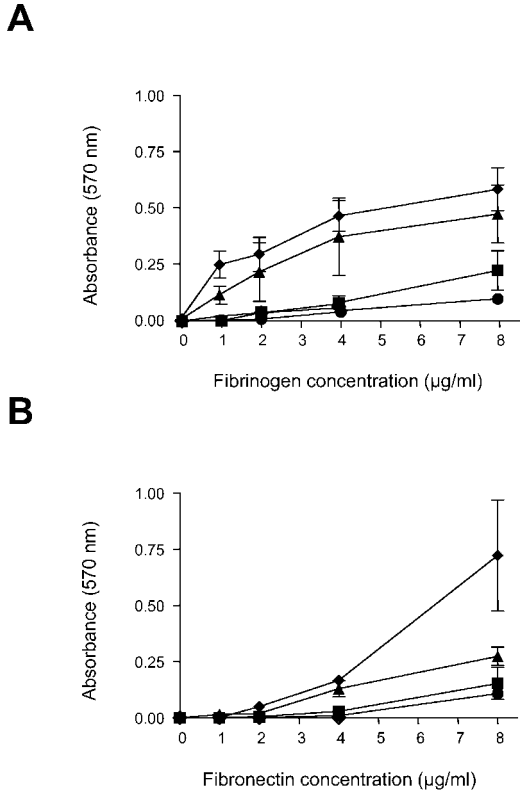

FIG. 3. Bacterial adherence of BB255 and its derivatives to increasing concentrations of immobilized fibrinogen (A) and immobilized fibronectin (B). Strains used were BB255 (squares), IK181 (BB255 Δ*rsbUVWsigB*, circles), GP268 (BB255 *rsbU*$^+$, triangles), and MB138 [BB255 *rsbW7*(Am), diamonds].

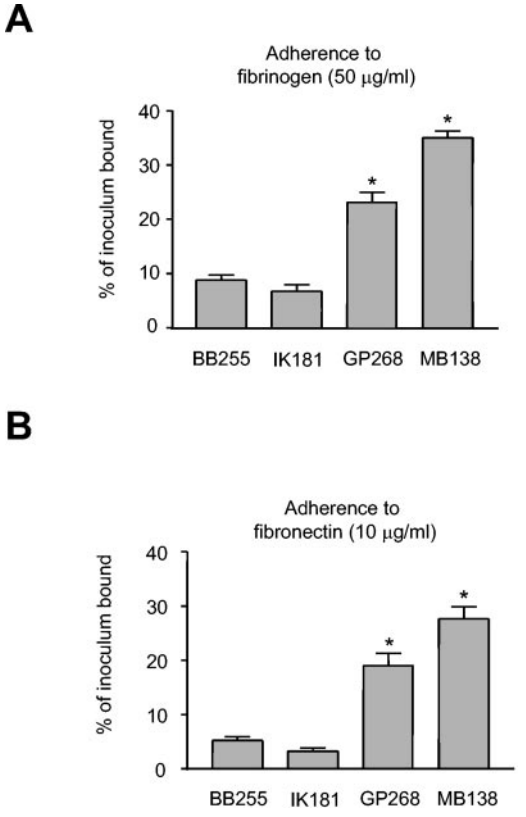

FIG. 4. Bacterial adherence of BB255 and its derivatives to immobilized fibrinogen (50 μg/ml) (A) or immobilized fibronectin (10 μg/ml) (B). Adherence was expressed as the percentage of bacteria bound to the immobilized host cell matrix proteins from an initial inoculum of 10^4 CFU.

the σ^B-activity-defective strains, illustrating the importance of ClfA and FnbA for the capacity of *S. aureus* to adhere to these host cell matrix proteins.

Adherence of *S. aureus* to platelet-fibrin clots. Since cardiac vegetations contain numerous proteins and platelet factors, which were not present in the in vitro ligand assay described above, the adherence experiments were repeated with platelet-fibrin clots, which more closely mimic cardiac vegetations of recovering damaged valves (39). Adherence of the σ^B-activity-producing strain GP268 to platelet-fibrin clots was higher than that of BB255 and its Δ*rsbUVWsigB* mutant, IK181, and was further increased in the σ^B overproducer MB138 ($P < 0.05$), indicating a positive correlation of σ^B activity with the capacity of *S. aureus* to adhere to platelet-fibrin clots (Fig. 5).

Experimental endocarditis. Binding of *S. aureus* to platelets has been considered crucial for the development of infective endocarditis by affecting vegetation formation and septic embolization (50, 51). Additionally, several in vivo studies have implicated ClfA, ClfB, and FnbA as pathogenic factors in experimental *S. aureus* infection (14, 40, 44, 52). Thus, the σ^B-activity-associated increase in binding of *S. aureus* to immobilized fibrinogen, fibronectin, and platelet-fibrin clots suggests that σ^B activity is likely to facilitate the development of infective endocarditis as well. This hypothesis was tested in a rat catheter-induced aortic vegetation model of infection. As in previous experiments (14, 40), the rate of infection 16 h postinoculation was inoculum dependent (Fig. 6). The BB255 derivatives caused endocarditis in 20 to 40% and 90 to 100% of rats at low and high inocula, respectively. No statistically significant difference in infectivity could be observed among any of the strains analyzed ($P > 0.05$), even though the σ^B-activity-

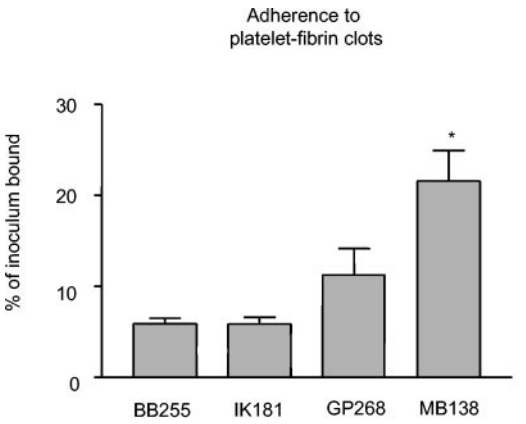

FIG. 5. In vitro adherence of *S. aureus* organisms to platelet-fibrin clots. The adherence ratio was expressed as a function of the original inoculum (see Materials and Methods). The results represent the means and standard deviations for 12 independent determinations. Asterisk, $P < 0.05$ versus all the other groups.

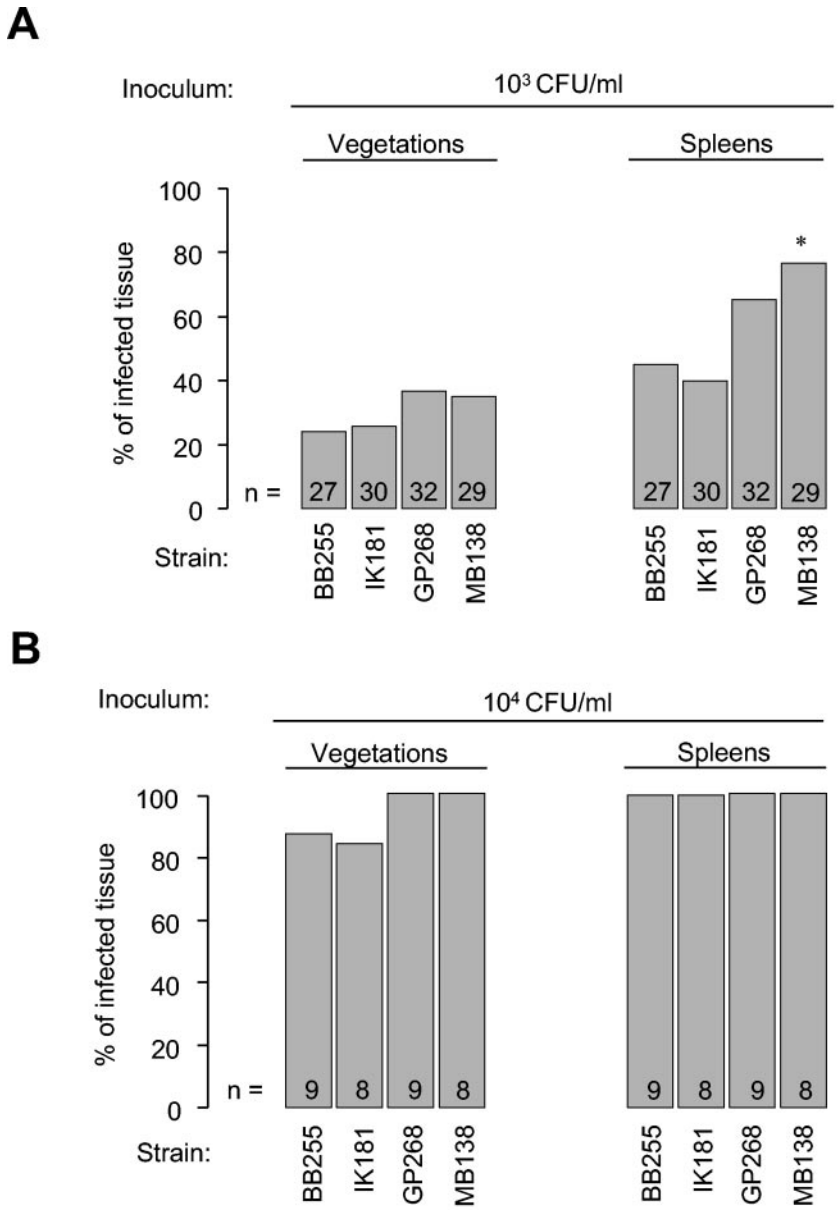

FIG. 6. Infectivity titer determination for the various test organisms in the rat model of experimental endocarditis 16 h postinoculation. The figure shows a compilation of results from six separate experiments. (A) An inoculum size of 10^3 CFU was used to obtain results in four experiments. Pooled results are shown. In two experiments, the rats were challenged with bacterial inocula of 2×10^3 to 3×10^3 CFU/ml. In the two other experiments, the rats were challenged with 4×10^3 6 × to 10^3 CFU/ml. The results were similar with both inocula. (B) An inoculum size of 10^4 CFU was used to obtain results in two experiments. The columns indicate the percentage of positive vegetations and spleen cultures. The number of animals per group (*n*) and the *S. aureus* strain are indicated at the bottom of the columns. Statistical differences were evaluated by the χ^2 test with the Yates correction. *, $P < 0.05$ compared with BB255 and IK181.

producing strains tended to cause slightly more endocarditis than did BB255 or its Δ*rsbUVWsigB* mutant (Fig. 6).

The bacterially challenged animals were also analyzed for positive spleen cultures (Fig. 6). In general, spleen cultures were positive more often than aortic cultures were. This demonstrated that the animals had been appropriately inoculated and suggested that spleen colonization was not necessarily dependent on valve infection. Importantly, in contrast to aortic vegetations, the frequency of positive spleen cultures correlated with increased σ^B activity (Fig. 6) and was most pronounced in rats challenged with the σ^B overproducer MB138 ($P < 0.05$). All spleen cultures became positive as the inoculum size was increased 10-fold (Fig. 6B). Although spleen colonization at 16 h after inoculation is primarily indicative of inoculation and does not necessarily indicate a spleen infection, it might become relevant in terms of the in vivo survival capacity of the bacteria, in combination with the respective bacterial density that is reached in this organ (see below).

Analysis of the bacterial densities obtained after 16 h revealed that all animals had comparable bacterial densities in

TABLE 2. Bacterial densities in vegetations and spleens of rats that developed experimental endocarditis 16 h after bacterial challenge

Bacterial strain	Median bacterial density[a] (CFU/g) (range) with inoculum of:			
	10^3 CFU		10^4 CFU	
	Vegetations	Spleens	Vegetations	Spleens
BB255	5.63 (4.51–6.22)	1.74 (1.57–2.47)	5.80 (3.67–8.57)	2.99 (2.35–4.29)
IK181	5.61 (3.52–7.88)	1.80 (1.05–2.36)	5.78 (3.01–7.36)	2.73 (2.33–3.53)
GP268	6.09 (2.40–7.20)	2.05 (1.53–2.86)	5.53 (3.98–8.87)	2.98 (2.50–4.59)
MB138	6.18 (4.28–7.93)	2.10 (1.60–3.30)†	7.85 (5.93–8.41)‡	3.28 (3.05–3.51)*

[a] †, $P < 0.05$ versus BB255 and IK181; ‡, $P < 0.05$ versus all the other groups; *, $P < 0.05$ versus IK181.

their aortic vegetations at low inocula ($P > 0.05$). However, at high inocula, the rats inoculated with the σ^B overproducer MB138 had significantly higher vegetation bacterial densities ($P < 0.05$) than did those in all other groups (Table 2). Additionally, the bacterial densities of MB138 within colonized spleens were significantly higher ($P < 0.05$) than those of BB255 and IK181 when the animals were challenged with a low inoculum (Table 2). Since all strains analyzed displayed similar abilities to grow under in vitro conditions, the higher vegetation bacterial densities observed for MB138 after 16 h was unlikely to be due to an increase in growth rate and thus may reflect an abated clearance of the σ^B-overproducing strain by host defense mechanisms at this site. Spleens are known to display a massive recruitment of polymorphonuclear neutrophils (PMNs), which are considered to fulfill major functions in the host defense system (13). Several laboratories have shown that σ^B activity significantly reduces the sensitivity of *S. aureus* to oxygen radicals and hydrogen peroxide (19, 24, 30), which are released by PMNs to challenge the pathogenic bacteria. Interestingly, SarA influences the capacity of *S. aureus* to be internalized by PMNs and to survive inside of PMNs (20). The findings that challenging rats with MB138 resulted in statistically significantly larger numbers of positive spleen cultures and higher bacterial densities 16 h postinoculation than did challenging them with BB255 or IK181, combined with the observation that σ^B-activity-possessing strains produce larger quantities of *sarA*-containing transcripts, suggest that σ^B activity may indeed contribute to the survival capacity of *S. aureus* under in vivo conditions during the early stages of infection, especially at sites with a high PMN content.

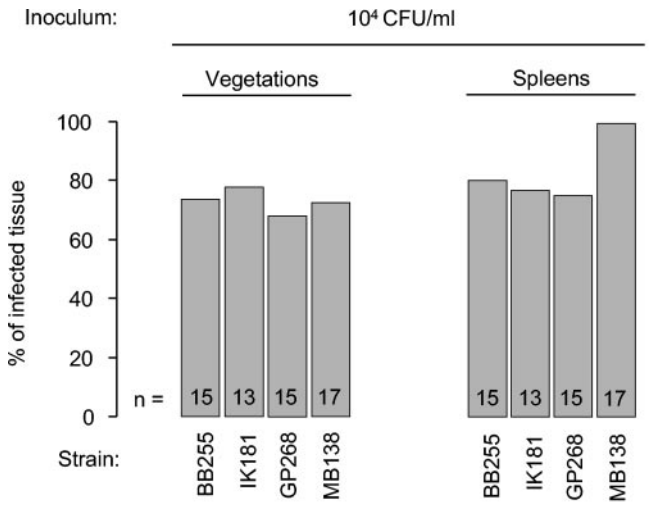

FIG. 7. Infectivity titer determination in the rat model of experimental endocarditis 72 h after inoculation with an inoculum size of 10^4 CFU. The figure shows a compilation of results of separate experiments. Details are as in Fig. 6.

To monitor the evolution of the infection with time, rats were challenged with high inocula and were sacrificed 72 h after bacterial inoculation. By 72 h postinoculation (Fig. 7), the rates of aortic infection slightly decreased relative to the rates at 16 h but remained similar between groups ($P > 0.05$). The number of CFU recovered in infected vegetations of rats inoculated with either BB255, IK181, or GP268 strains increased relative to the number recovered at 16 h and were larger (although not statistically significantly larger) than the number of CFU recovered from vegetations of rats inoculated with MB138, which decreased relative to the number observed by 16 h.

By 72 h postinoculation, the rates of spleen infection of rats inoculated with either BB255, IK181, or GP268 slightly decreased relative to the rates observed at 16 h (Fig. 6). In contrast, the frequency of positive spleen cultures remained unchanged (100%) in animals that were challenged with MB138. However, the difference in spleen infection frequency between MB138 and the other derivatives tested was not statistically significant. Surprisingly, while the number of CFU obtained from spleens of rats inoculated with either BB255, IK181, or GP268 increased relative to the number obtained 16 h, the same was not observable for MB138 (Table 3). The number of CFU obtained from the spleens of MB138-inoculated animals after 72 h remained more or less unchanged relative to the number obtained after 16 h and was significantly smaller than the number of CFU recovered from the spleens of BB255, IK181, or GP268-infected animals ($P < 0.05$). The observed decrease in bacterial densities in both vegetations and spleens by 72 h suggests that high σ^B activity negatively

TABLE 3. Bacterial densities in infected vegetations and spleens of rats 72 h after bacterial challenge with an inoculum of 10^4 CFU

Bacterial strain	Median bacterial density[a] (CFU/g) (range) in:	
	Vegetations	Spleens
BB255	8.75 (6.30–9.84)	5.44 (4.49–5.60)†
IK181	8.32 (3.37–9.35)	5.17 (1.78–5.60)†
GP268	9.19 (5.75–9.57)	5.15 (3.05–5.64)†
MB138	6.03 (3.11–9.48)	3.43 (1.96–5.56)

[a] †, $P < 0.05$ versus MB138.

influences the ability of *S. aureus* to develop high cell densities at the infection site during later stages of infection.

The in vivo data presented here demonstrate that σ^B activity has little to no effect on the development of infective endocarditis, irrespective of its ability to positively influence the expression of *clfA* and *fnbA*, somehow contradicting previous publications showing that inactivation of *clfA* or *fnbA* had small but significant impacts on the infectivity of *S. aureus* (40, 52). It is worth noticing that Xiong at al. (55), who investigated the impacts of *sar* and *agr* mutations on *fnbA* expression and on the fibronectin adherence capacity under in vitro and in vivo conditions, found that mutations of *sarA* and/or *agr* resulted in a marked effect on *fnbA* expression but had no statistically significant impact on infectivity in an experimental rabbit endocarditis model. It becomes clear from the transcription and adhesion data shown here that *S. aureus* is still able to produce significant amounts of fibrinogen- and fibronectin-binding proteins even in the absence of σ^B activity. The amounts of *clf*- and *fnb*-encoding transcripts produced by the *sigB* mutant strains seem to be sufficient to allow the pathogen to bind to the host cell matrix proteins in vivo, giving a simple explanation of why mutations of *clfA* or *fnbA* decrease the infectivity of *S. aureus* but *sigB* does not. Moreover, our in vivo findings are in agreement with previous findings demonstrating that mutations in *sigB* had no significant effect on the virulence of *S. aureus* in four different animal models (6, 24, 41).

Considering that σ^B-dependent promoters are present in the upstream regulatory regions of many virulence-associated loci, it is nevertheless surprising that any attempt to demonstrate an influence of σ^B on the virulence of *S. aureus* in an animal model has failed to date. Based on the findings that the expression of various virulence-associated factors is influenced by σ^B activity (3, 4, 18, 19, 24, 30, 42) but that no influence on infectivity has been demonstrated so far, we have to conclude that σ^B is more likely to be a modulator of virulence gene expression and not a virulence determinant by itself. Thus, one possible function of σ^B on *S. aureus*-host interactions might be the global regulation of factors associated with virulence in a way (i) promoting adhesion of the pathogen to host cells, i.e., by upregulation of MSCRAMMS, and (ii) allowing the organism to persist at the attachment site without harming the host tissue, i.e., by down-regulation of exoprotein production, thereby reducing the inflammatory response caused by the host defense system. Such a function of σ^B would fit with our observations that σ^B had a positive impact on the ability of *S. aureus* to reach high cell densities during the early stages of infection while having a negative influence on the capacity of the pathogen to develop high cell densities during later stages of infection. Further support is given by the recent findings by Bischoff et al. (4), who showed that σ^B had a negative influence on the expression of various excreted virulence factors, such as proteases, lipases, and hemolysins, which all are supposed to be important for the ability of *S. aureus* to evade the site of infection, once the infection has been established. While promoting adhesion during early stages, σ^B activity may decrease the ability of the pathogen to evade the primary site of infection during later growth stages, thus decreasing its capacity to damage the host cell tissue, and to establish new sites of infection.

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